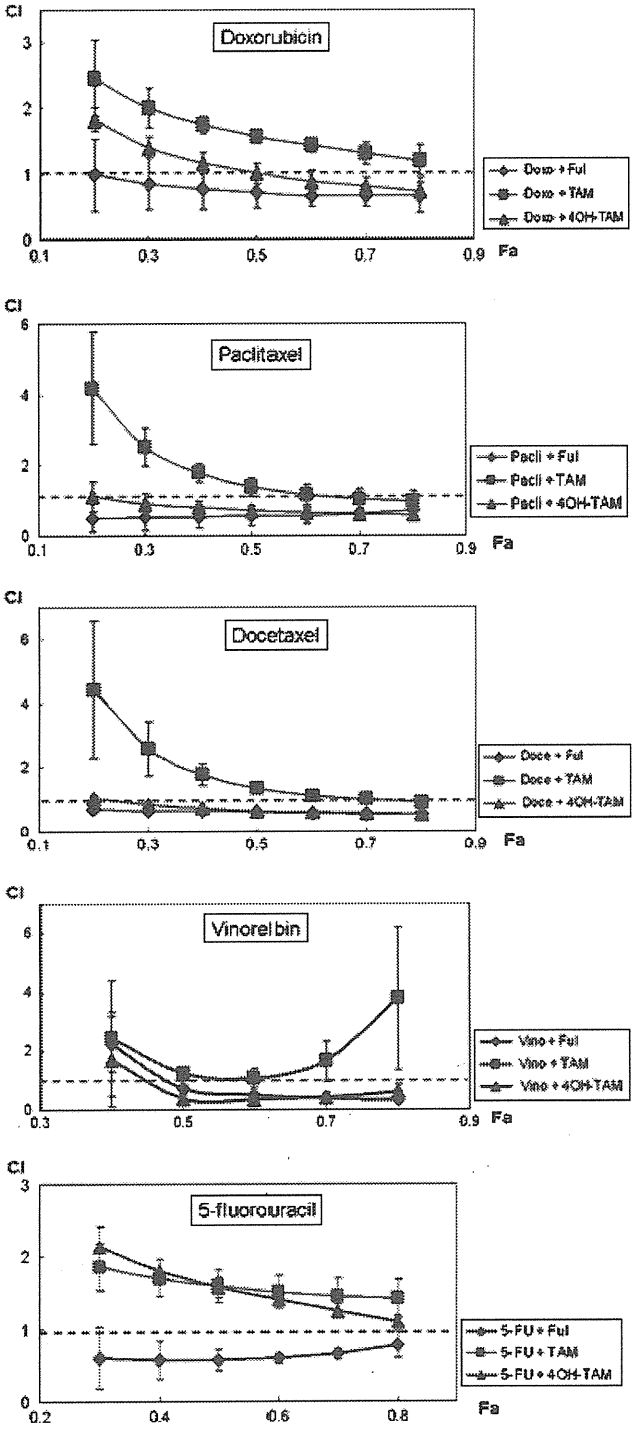

Fig. 1. Effects of combination treatment with chemotherapeutic agents and hormone drugs in breast cancer. Fulvestrant (Ful) showed a synergistic effect with all five chemotherapeutic agents (doxorubicin [Doxo], paclitaxel [Pacli], docetaxel [Doce], vinorelbine [Vino], and 5-fluorouracil [5-FU]) regardless of the fulvestrant dose. Tamoxifen (TAM) had an antagonistic effect with all chemotherapeutic agents. 4-Hydroxytamoxifen (4-OH-TAM) showed an antagonistic effect with doxorubicin and 5-fluorouracil, but a synergistic effect with taxanes and vinorelbine. The antagonistic effects of 4-hydroxytamoxifen with doxorubicin and 5-fluorouracil were weaker than those of tamoxifen. Data are shown for MCF-7 cells. CI, combination index; Fa, fraction affected.

Table 2. Combination index (CI) values of chemotherapeutic agents and hormone drugs in two breast cancer cell lines

	CI values at		
	ED_{50} (average ± SD)	ED_{75} (average ± SD)	*r*
MCF-7 cells			
Doxorubicin			
Fulvestrant	0.71 (0.22)	0.66 (0.20)	0.99
Tamoxifen	1.60 (0.06)	1.26 (0.20)	0.97
4-Hydroxytamoxifen	1.01 (0.16)	0.77 (0.13)	0.97
Paclitaxel			
Fulvestrant	0.55 (0.28)	0.66 (0.12)	0.97
Tamoxifen	1.38 (0.26)	1.00 (0.28)	0.97
4-Hydroxytamoxifen	0.72 (0.12)	0.62 (0.08)	0.97
Docetaxel			
Fulvestrant	0.59 (0.17)	0.53 (0.14)	0.98
Tamoxifen	1.35 (0.11)	0.91 (0.16)	0.97
4-Hydroxytamoxifen	0.65 (0.02)	0.55 (0.03)	0.97
Vinorelbin			
Fulvestrant	0.66 (0.13)	0.38 (0.07)	0.98
Tamoxifen	1.22 (0.23)	2.36 (1.21)	0.97
4-Hydroxytamoxifen	0.40 (0.15)	0.51 (0.22)	0.98
5-Fluorouracil			
Fulvestrant	0.57 (0.07)	0.71 (0.25)	0.98
Tamoxifen	1.59 (0.23)	1.44 (0.29)	0.99
4-Hydroxytamoxifen	1.58 (0.12)	1.12 (0.09)	0.98
ZR75-1 cells			
Doxorubicin			
Fulvestrant	0.66 (0.16)	0.70 (0.66)	0.98
Tamoxifen	1.72 (0.39)	1.19 (0.22)	0.98
4-Hydroxytamoxifen	1.41 (0.11)	1.15 (0.17)	0.99
Paclitaxel			
Fulvestrant	0.45 (0.22)	0.56 (0.32)	0.97
Tamoxifen	1.42 (0.35)	1.04 (0.09)	0.98
4-Hydroxytamoxifen	0.92 (0.04)	0.95 (0.10)	0.97
Docetaxel			
Fulvestrant	0.53 (0.31)	0.60 (0.20)	0.98
Tamoxifen	1.33 (0.37)	1.11 (0.27)	0.97
4-Hydroxytamoxifen	1.09 (0.12)	1.04 (0.07)	0.97
Vinorelbin			
Fulvestrant	0.46 (0.04)	0.56 (0.09)	0.99
Tamoxifen	1.30 (0.25)	1.48 (0.38)	0.98
4-Hydroxytamoxifen	0.68 (0.12)	0.82 (0.12)	0.97
5-Fluorouracil			
Fulvestrant	0.74 (0.04)	0.71 (0.09)	0.98
Tamoxifen	1.82 (0.69)	1.45 (0.29)	0.99
4-Hydroxytamoxifen	1.05 (0.11)	0.86 (0.08)	0.98

The values are the average of four independent experiments, with the SD given in parentheses. Fulvestrant showed a synergistic effect with all five chemotherapeutic agents regardless of the fulvestrant dose. Tamoxifen showed an antagonistic effect with all the chemotherapeutic agents. 4-Hydroxytamoxifen showed an antagonistic effect with doxorubicin and 5-fluorouracil, and a synergistic effect with taxanes and vinorelbine. These trends were found in both cell lines. *r*, linear correlation coefficient of the median-effect plot.

by fulvestrant. The MRP1 level was increased in a serum-free medium compared with control, decreased by 17-β estradiol, and increased by fulvestrant (Fig. 2A). Tamoxifen and 4-hydroxytamoxifen both increased MAPT and Bcl2 levels and decreased the MRP1 level (Fig. 2B). These results indicated that the chemoresistant factors Bcl2, MRP1, and MAPT were influenced by ER and modulated by hormone drugs. The effects of fulvestrant on these factors were opposite to those of 17-β

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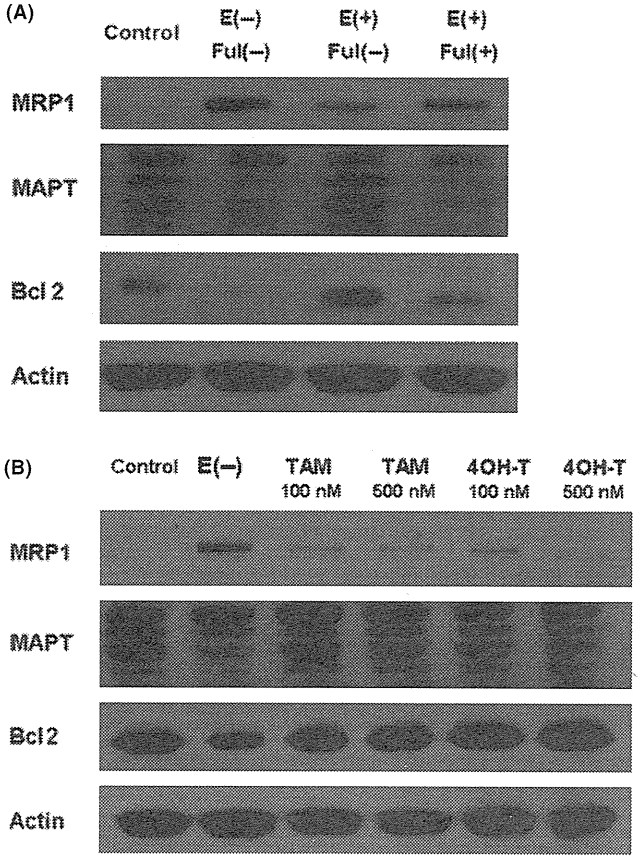

Fig. 2. Influence of hormone drugs on expression of chemoresistant factors in breast cancer. (A) Cells were seeded in a serum-free medium and incubated for 24 h, then cultivated in a medium containing 17-β estradiol (E; 1.0 nM) alone, fulvestrant (Ful; 100 nM) alone, or a combination of the two for 72 h, then harvested for Western blot analysis. The levels of microtubule-associated protein tau (MAPT) and Bcl2 were increased by 17-β estradiol and decreased by fulvestrant. The multidrug resistance-associated protein 1 (MRP1) level was increased in serum-free medium compared with control, decreased by 17-β estradiol, and increased by fulvestrant. (B) Cells initially treated as above were cultivated in a medium containing tamoxifen (TAM; 100 or 500 nM) or 4-hydroxytamoxifen (4-OH-T; 100 or 500 nM) for 72 h, then harvested for Western blot analysis. The levels of MAPT and Bcl2 were increased and the level of MRP1 was decreased by both tamoxifen and 4-hydroxytamoxifen.

estradiol, and also differed from the effects of tamoxifen and 4-hydroxytamoxifen. The estrogen-like agonist activity of 4-hydroxytamoxifen was similar to that of its parent drug.

Combination effects of fulvestrant and chemotherapeutic agents *in vivo*. To examine the compatibility of fulvestrant with chemotherapeutic agents, combination effects with docetaxel and doxorubicin were examined *in vivo*. With docetaxel, mice were randomized into four groups that received: no treatment (control group, $n = 8$); docetaxel given i.p. (20 mg/kg; $n = 10$) on days 1 and 8; fulvestrant given s.c. (5 mg/body; $n = 10$) on days 1 and 8; and docetaxel and fulvestrant (20 and 5 mg/body; $n = 10$) on days 1 and 8. For doxorubicin, the mice were also randomized into a non-treatment control group ($n = 8$) and groups given doxorubicin i.p. (2 mg/kg; $n = 10$) on days 1, 8, and 15; fulvestrant s.c. (5 mg/body; $n = 10$) on days 1, 8, and 15; and a combination of docetaxel and fulvestrant (20 and 5 mg/body; $n = 10$) on days 1, 8, and 15. Combination treatment with docetaxel and fulvestrant had a synergistic effect on

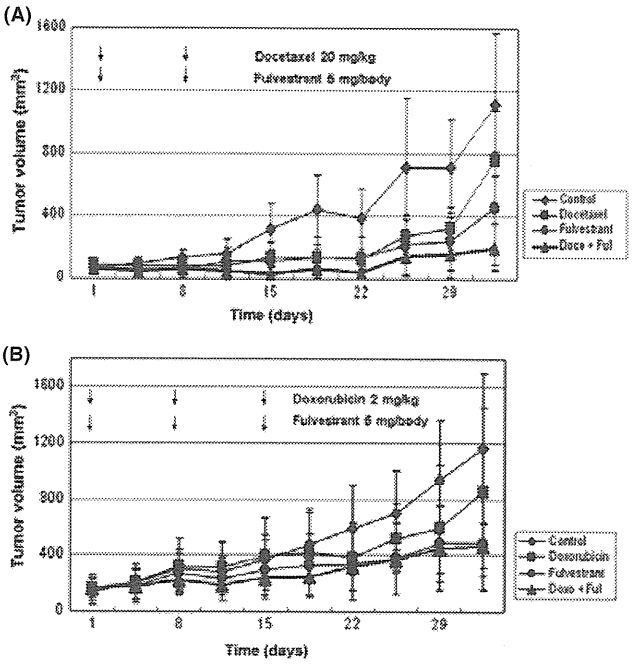

Fig. 3. Combination effect of fulvestrant and docetaxel *in vivo*. Xenografts were created using MCF-7 cells transplanted into BALB/c nu/nu mice. Tumor sizes were evaluated after the mice were treated with the chemotherapeutic agent (docetaxel [Doce] or doxorubicin [Doxo]) alone, fulvestrant (Ful) alone, or combinations of a chemotherapeutic agent and fulvestrant. (A) Combination treatment with docetaxel and fulvestrant had a synergistic effect on tumor growth. Tumor growth was significantly inhibited compared with docetaxel alone ($P = 0.0017$, Student's *t*-test) and fulvestrant alone ($P = 0.021$). (B) Tumor growth was also significantly inhibited by combination treatment with doxorubicin and fulvestrant compared with doxorubicin alone ($P = 0.0396$, *t*-test), but there was no significant difference in tumor size between the combination treatment and fulvestrant alone ($P = 0.9385$). Thus, combination treatment with doxorubicin and fulvestrant did not show a synergistic effect.

tumor growth. Tumor growth was significantly inhibited by combination treatment with docetaxel and fulvestrant compared with docetaxel alone ($P = 0.0017$, Student's *t*-test) and fulvestrant alone ($P = 0.021$). Tumor growth was also significantly inhibited by combination treatment with doxorubicin and fulvestrant compared with doxorubicin alone ($P = 0.0396$, *t*-test), but there was no significant difference in tumor size between combination treatment and fulvestrant alone ($P = 0.9385$). Thus, combination treatment with doxorubicin and fulvestrant did not show a synergistic effect (Fig. 3).

Discussion

The results of this study indicate that combination chemotherapy and endocrine therapy may offer a new approach to treatment of ER-positive breast cancer. Good compatibility of fulvestrant with chemotherapeutic agents *in vitro* and *in vivo* was shown, and the marked utility of combination treatment with docetaxel and fulvestrant *in vivo* may have an impact on current ideas on combination chemotherapy and endocrine therapy. These ideas are based on data showing that tamoxifen has an antagonistic effect when given with chemotherapeutic agents.[17–22] In a previous study, we reported that a combination of a taxane and tamoxifen had an antagonistic effect that was partly caused by the estrogen-like agonist activity of tamoxifen.[11] In contrast, fulvestrant showed a synergistic effect with

all five chemotherapeutic agents used in the current study. This may partly be due to fulvestrant downregulating ER and the chemoresistant factors modulated by ER, thereby enhancing the effect of chemotherapy.[11,23–25] Therefore, modern hormonal drugs can have different effects to those of tamoxifen and may show different activities in combination with chemotherapy. The results of this study clearly show that fulvestrant is superior to tamoxifen and 4-hydroxytamoxifen in combination with chemotherapeutic agents.

Tamoxifen is metabolized *in vivo* and the parent molecule and its metabolites have different effects on ER. Therefore, we examined the effects of tamoxifen and those of a metabolite (4-hydroxytamoxifen) in combination with chemotherapeutic agents to evaluate the effects of combination therapy of tamoxifen and chemotherapeutic agents under conditions closer to the clinical situation. Tamoxifen and 4-hydroxytamoxifen also showed some interesting differences. 4-Hydroxytamoxifen had antagonistic effects with doxorubicin and 5-fluorouracil, but these were weaker than those of tamoxifen. With other chemotherapeutic agents, 4-hydroxytamoxifen had synergistic effects, while tamoxifen showed antagonistic effects. However, the reasons for the different effects of the two agents in combination with anticancer drugs were not examined in this study.

Overall, our results show the superiority of fulvestrant in combination with chemotherapeutic agents, and the relative uncertainty of using tamoxifen in this manner. A combination of fulvestrant and chemotherapy (especially with taxanes) could be worth examining for ER-positive breast cancer, which shows low sensitivity to current chemotherapy. Such combination therapy may be effective for further improvement of the prognosis of patients with this form of breast cancer.

In conclusion, tamoxifen and its active metabolite (4-hydroxytamoxifen) had different effects on breast cancer cell lines in combination with chemotherapeutic agents. Fulvestrant downregulated the ER, reduced the levels of ER-regulated chemoresistant factors such as Bcl2 and MAPT, and had good compatibility with all evaluated chemotherapeutic agents *in vitro* and *in vivo*. These results suggest that a combination of fulvestrant and chemotherapy could be effective for treatment of ER-positive breast cancers.

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Disclosure Statement

The authors have no conflict of interest.

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Original Article

Clinical Efficacy of Capecitabine and Cyclophosphamide (XC) in Patients with Metastatic Breast Cancer

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Combined low-dose therapy of oral capecitabine (Xeloda) and cyclophosphamide (XC) has been demonstrated to be useful for long-term control of lesions in patients with metastatic breast cancer (MBC) and is aimed at symptomatic alleviation and prolongation of survival. Here, a retrospective review was conducted of MBC patients administered XC at the Okayama University Hospital (OUH), to evaluate responses to XC, adverse events and time to progression (TTP). Twenty patients with MBC received XC between 2006 and 2009. With the exception of 2 elderly patients who were over the age of 70 at the initial examination, all of the patients had received prior treatment with an anthracycline and/or a taxane. No complete response (CR) cases were observed, but partial response (PR) was achieved in 6 patients (30%) and SD in 9 (45%), of whom 5 (20%) sustained SD status for ≥12 months. The median TTP was 6 months (range: 3-27 mo.). Three patients developed Grade 3 adverse events (diarrhea, nausea and stomatitis), but no other patients developed adverse reactions causing interruption of the therapy. XC was safe even in previously treated and elderly MBC patients; moreover, it yielded remarkable clinical responses.

Key words: metastatic breast cancer, metronomic, chemotherapy

With advances in the development of new drugs in recent years, an expanded repertoire of pharmacotherapeutic strategies has become available for breast cancer. As molecular-targeting drugs become more widespread, pharmacotherapy has become increasingly more effective but also more complex. The selection of drugs must be based on results of individual drug sensitivity assessments. In the case of breast cancer treatment, in particular, the most suitable therapeutic regimens should be selected not only based on assessment of the indication for hormone therapy or for the molecular-targeting drug trastuzumab by determining the estrogen receptor (ER) and HER2 expression status, but also by taking into account the tumor characteristics, such as the malignancy grade, extent of lymph node metastasis, and sites of distant metastasis through translational research which has been applied extensively in recent years. Risk factors such as adverse reactions, cost, and the social environment of the patients are also of importance in this determination. In particular, treatment for recurrent carcinoma of the breast is still aimed primarily at prolongation of survival and alleviation of symptoms rather than at cure of the malignancy, so that the weight of each of these factors diverges widely from that during the consideration of adjuvant chemotherapy, which is aimed at cure. It sum, theoretical and clinical evidence-based evaluation

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of the risk *versus* benefit of various therapeutic strategies on a patient-by-patient basis has been increasingly expected in recent years.

Patients with recurrent breast cancer present with diverse symptoms depending on the site(s) of the metastatic lesions and exhibit anxiety about exacerbation of the condition. Many are elderly and inevitably require familial support. Drugs that can be used safely and effectively for prolonged periods of time in such patients are still very few.

This study was undertaken as a retrospective evaluation of the clinical efficacy of and adverse reactions to XC therapy, one of the treatment options employed for recurrent breast cancer, consisting of capecitabine (Xeloda, X) and cyclophosphamide (C), both available in oral formulations.

Patients and Methods

Patients. A retrospective analysis was performed of patients with MBC who received combined capecitabine and cyclophosphamide therapy (XC) between December 2004 and March 2009 at Okayama University Hospital (OUH). The patient population was identified from a database at the Division of Breast and Endocrine surgery. They were followed up until death or, if still alive, until their last visit up to March 2009.

Baseline evaluation included clinical examination, chest X-ray, CT, nuclear bone scan, and biochemical and hematological tests. The complete blood count and biochemical tests were repeated every 21 days. The best response in each patient was assessed according to the WHO criteria. Complete response (CR) was defined as disappearance of all clinical and radiographic evidence of the tumor as assessed on two occasions at least 4 weeks apart. Partial response (PR) was defined as a 30% or greater decrease in the sum of the maximum perpendicular diameters of measurable lesions. Stable disease (SD) was defined as a less than 30% decrease but greater than 25% increase in the sum of the bi-perpendicular diameters of measurable lesions and the absence of the appearance of new lesions; these conditions had to be maintained for at least 12 weeks to be labeled as SD, and SD maintained for over 40 weeks was defined as prolonged SD. Progressive disease (PD) was defined as a greater than 25% increase in the sum of bi-perpendicular diameters of measurable lesions, or the appearance of new lesions. The clinical benefit rate was defined as the proportion of patients in whom a CR, PR or prolonged SD was achieved. The National Cancer Institute common terminology criteria for adverse events (CTCAE) version 2.0 [1] were adopted to determine the toxicity of the treatment.

Evaluation of pathological factors. Surgical specimens were sectioned at 7–10 mm for evaluation of the pathological response by pathologists. Expression levels of ER (1D5, Dako Cytomation, Glostrup, Denmark), PgR (1A6, Novocastra), and HER2 (HercepTest®, Dako Cytomation) were examined by immunohistological staining. The ER and PgR status was labeled as positive when greater than 10% of the cancer cell nuclei exhibited positive staining, regardless of the staining intensity. HER2 expression was scored as follows: (0), no positive cell staining; (1+), slightly positive in more than 10% of the cancer cells; (2+), moderately positive in more than 10% of the cancer cells; and (3+), markedly positive in more than 10% of the cancer cells. Immunohistochemistry (IHC) scores of (2+) or (3+) were defined as HER2-positive.

Treatment. Capecitabine (1,600 or 2,400 mg/day) and cyclophosphamide (100 mg/day) (XC) were administered orally twice daily for 2 weeks, followed by a week of treatment cessation. Treatment was continued until disease progression, appearance of unacceptable adverse events or withdrawal of the patient's consent. In the case of Grade 2 or worse toxicity, XC administration was interrupted and resumed only after the toxicity had resolved entirely or improved to Grade 1.

The time to progression (TTP) was calculated from the day of commencement of XC administration until the day of documented progression. Overall survival (OS) was calculated from the start date of XC therapy to the date of death from any cause. TTP and OS were analyzed according to Kaplan-Meier estimates.

Results

Twenty MBC patients received XC therapy between December 2004 and March 2009 at OUH. Three of these patients were still receiving XC at the last follow-up. Table 1 shows the patient characteristics. The median age was 56 (29–83) years. The

Eastern Cooperative Oncology Group (ECOG) performance status of the patients was <2 in all patients. The site of metastatic disease was the bone and/or soft tissue in 5 patients (25%) and a visceral site(s) (lung, liver, brain and pleura) in 15 patients (75%). Table 2 shows the chemotherapy regimens that the patients had received prior to the XC therapy. The median number of chemotherapy regimens used before the XC regimen was 2 (0-5). All except 2 patients who were older than 70 years old had received an anthracycline and/or a taxane. Three patients (15%) had received vinorelbine and 5 (25%) had received a 5FU derivative prior to the XC therapy. Prior oral formulations received included CMF (2 patients), capecitabine alone (2 patients) and S-1 (1 patient). Eleven (55%) patients were ER-positive and had received hormone therapy prior to the XC treatment. Three patients (15%) with HER2-positive disease had received trastuzumab in combination with a taxane or vinorelbine before the XC treatment, and 2 had received XC with trastuzumab.

The response rate (RR) was 30%, with none of the patients showing clinically complete response (CR) and 30% (6/20) showing PR. Nine patients (45%) showed SD, and prolonged SD with continued XC administration for more than 12 months was observed in 4 (20%) patients (Table 3). The overall clinical benefit rate (CR, PR and prolonged SD) was 50% (10/20). Six out of the 10 patients with clinical benefit had visceral involvement (liver, 4; lung, 2), and 75% (3/4) of the patients who showed prolonged SD had bone metastasis only. Six out of the 10 patients were ER-positive and one was HER2-positive. Four of the patients had triple-negative breast cancer, being negative for ER, PgR and HER2, and the response rate to XC in these patients was relatively high (PR 2, prolonged SD 1 and SD 1). Two of the patients who had received capecitabine alone before the XC treatment showed SD. The median TTP was 6 months (range, 1-27 months; Fig. 1). The median OS from the start of treatment for the metastases was 38 months (range, 9-86 months), and 6 patients (30%) were still alive at the last follow-up.

Overall, the XC regimen was relatively well-tolerated. Table 4 shows the adverse events that were encountered in the patients treated with XC. Grade 3 toxicities were observed in 3 patients (diarrhea, 2; nausea, 1; stomatitis, 1). There was no case of hand-foot syndrome or febrile neutropenia, and none of the XC-related adverse events were fatal. The most frequent reason for treatment discontinuation was disease progression (14 patients, 82%). The XC treatment was discontinued and other chemotherapy started in the 3 patients who showed Grade 3 adverse events. One of the 2 relatively older patients (73 and

Table 1 Patient characteristics

	No. of patients (n = 20)	% of patients
Median age (years; range)	56 (29-83)	
Metastatic site(s) involved		
Bone/soft tissue	5	25
Visceral	15	30
Histology		
Invasive ductal carcinoma	19	95
Invasive lobular carcinoma	1	5
Estrogen receptor status		
Positive	11	55
Negative	9	45
HER2/neu status		
Positive	5	25
Negative	15	75

Table 2 Prior chemotherapy

Prior chemotherapy	No. of patients (n = 20)	% of patients
No. of regimens used		
0/1/2/3/4/5	2/5/7/3/2/1	
Median	2 (0-5)	
Anthracycline	16	80
Taxane	14	70
Vinorelbine	3	15
Capecitabine	2	10
S-1	1	5
Trastuzumab	3	15

Table 3 Response rate

Response	No. of patients (n = 20)	% of patients
CR	0	0
PR	6	30
SD	9	45
Prolonged SD (>12 months)	4	20
PD	5	25

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

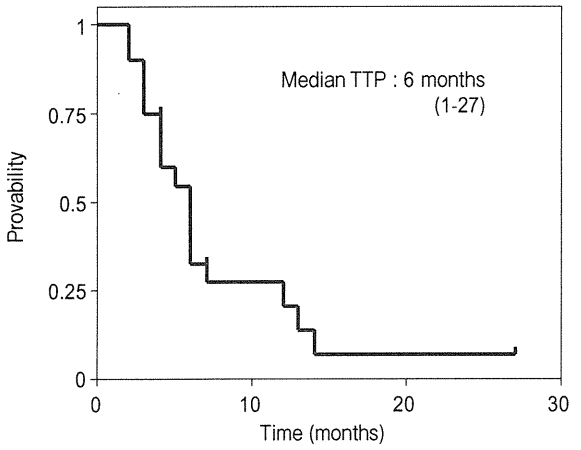

Fig. 1 Time to progression curve of metastatic breast cancer patients treated with Capecitabine and Cyclophosphamide (XC).

Table 4 XC-treatment-related adverse events

event	Grade 1/2 (%)	Grade 3 (%)
Diarrhea	0	1 (5)
Anorexia	1 (5)	0
Nausea/vomiting	0	2 (10)
Stomatitis	0	1 (5)
Generalized fatigue	1 (5)	0
Hand-foot syndrome	0	0

83 y.o.) who were administered XC as first-line chemotherapy showed PR and the other showed prolonged SD without the development of adverse events.

Discussion

Following its administration, capecitabine (X) is converted to its active form 5-FU by thymidine phosphorylase (TP), which occurs at high levels in tumor tissues, to exert its antitumor effect [2]. Disease control with X treatment alone has been reportedly achieved in 57–63% of patients with anthracycline- and taxane-refractory, recurrent breast cancer, with a median survival time of about 1 year [3–5]. The higher the level of TP activity in the tumor tissues, the greater the clinical benefit obtained with X therapy [6]; concomitant use of a drug(s) enhancing the TP activity in the tumor tissues may augment the therapeutic effect of this drug. It has been reported that besides paclitaxel, docetaxel and mitomycin C administration, cyclophosphamide administration is also associated with elevation of the intratumoral TP activity [7–10]. Several clinical trials of combined therapy with capecitabine and paclitaxel or docetaxel have been conducted, with good results, demonstrating the superiority of the combined therapeutic regimens [3, 4, 11–14]. In particular, a study designed to compare the results of treatment with X + docetaxel and docetaxel alone in patients with recurrent breast cancer previously treated with anthracyclines showed that the combined regimen was significantly superior in terms of the response rate and response duration, as well as the survival time [11]. Several clinical studies have been published on the usefulness of combined treatment with molecular-targeting therapeutic agents. Use of these drugs in the treatment of recurrent breast cancer, however, is still controversial, because of the significant adverse reactions and unusually high prices of these drugs, the as-yet insufficient assessment of the therapeutic responses, and the fact that the patient's disease status, social background, *etc.*, must be taken into account when prescribing them.

As for the adverse reactions to X, symptoms related to the gastrointestinal system and myelosuppression are relatively mild, while hand-foot syndrome has been reported to occur at a high frequency. In the present case series, none of the patients developed hand-foot syndrome. Myelosuppression may occur also as an adverse reaction to C but there was no patient in the present case series with febrile neutropenia or bone marrow suppression necessitating treatment discontinuation. In 3 patients (15%), adverse reactions necessitated discontinuation of treatment and a switch to other therapeutic regimens; in all 3 patients, the adverse reaction pertained to the gastrointestinal system. Measures to prevent these gastrointestinal reactions should be taken from the outset. In the present case series, with the patient age reaching up to 83 years, XC therapy proved to be safe, raising no concerns in terms of adverse reactions. Thus, XC therapy is a remarkably well tolerated and safe treatment. Furthermore, it is considered that XC therapy may be safely and effectively employed for the treatment of breast cancer in elderly subjects for whom a definitive treatment policy is yet to be established.

The advantage of orally available medications lies first in their milder adverse reactions and greater

patient compliance, which allow a good QOL to be maintained. As the aims of treatment in patients with recurrent breast cancer are prolongation of survival and alleviation of symptoms, combinations of drugs must be carefully chosen for a therapeutic strategy for maintaining patient QOL. Currently, treatment with capecitabine alone is undertaken in a number of patients with recurrent breast cancer, and this therapy is generally thought to be beneficial for maintenance of a good QOL. The XC therapy in the present series consisted of X in combination with a low dose of C. According to a report by Harvey V *et al.*, XC therapy was superior by 12.6% in terms of the clinical response to treatment with capecitabine alone [15]. In regard to adverse reactions, neutropenia associated with C was noted in the XC therapy group; however, there were no other significant intergroup differences. There has been no large-scale prospective study published to date, so that further investigation is warranted to clarify any differences in survival [7-8, 16]. In the present case series also, partial response were obtained in 30% of all the patients, including patients with a history of prior treatment. Furthermore, for the first time, it has been documented that 20% of the patients showed a prolonged duration of SD that was sustained for ≥1 year. These responses seem to be largely attributable to the metronomic therapeutic effect obtained with the use of the combined XC regimen [2, 7]. XC therapy, when viewed from these viewpoints, may yield responses as satisfactory as those of hormonal therapy, with a long-sustained QOL; therefore, its institution from an early stage of treatment is expected to be of significance especially in patients with breast cancer not showing adequate response to hormonal therapy.

In recent years, the development of an effective therapeutic strategy for dealing with basal-like carcinoma of the breast, which is recognized as a highly malignant type with an unfavorable prognosis, has drawn increasing attention [17]. This type of breast cancer has been shown to involve mutations of BRCA-1, so that genetic instability is likely implicated in its pathogenesis. Further, it has been documented as being refractory to currently available drugs, and no standard treatment has been established yet. There is the possibility, however, that alkylating agents such as C, which directly act upon DNA strands to cause inter-strand linking, may prove effective against it. Therefore, orally available C preparations are currently the focus of attention for the treatment of these breast tumors, and results of relevant studies are eagerly awaited. The present study data suggest the potential usefulness of XC therapy in the treatment of triple-negative breast carcinomas, in that the therapy yielded PR in 50% of the patients with triple-negative breast carcinoma, albeit there were only 4 such patients. However, there is no established therapeutic strategy using these drugs for the treatment of early drug-resistant triple-negative breast carcinomas. These treatment regimens continue to be instituted only after the standard use of anthracyclines and taxanes, as is the case with other types of breast cancer. Further investigations are needed to select the most effective treatment method with prior scrutiny of the tumor susceptibility to drugs. It seems very likely that XC therapy would serve as an important treatment alternative under these circumstances.

The therapeutic concept of inhibition of tumor growth by inhibiting peritumoral neomicrovascularization to produce a resting state of neoplastic growth is referred to as metronomic chemotherapy [18]. Laboratory studies have demonstrated the anti-neoangiogenic and antitumor effects of long-term administration of C at low doses [19]. This concept has been clinically applied, and reports have been appearing in the literature, including one study in which low-dose C was used concomitantly with hormonal therapy [20] and more recently, trials of combined treatment with molecular-targeting drugs that inhibit neovascularization, with the expectation of antiangiogenic effects [21-22]. There has been a growing trend of devising therapeutic strategies based on the same concept for other solid tumors as well as breast cancer [23]. The XC therapy reported herein also represents a regimen for metronomic chemotherapy aimed at long-sustained inhibition of tumor growth via combined use of low-dose C and X, which enhances the effect of the former. These therapeutic regimens may be said to be useful inasmuch as an anti-neoangiogenic effect can be expected from both regimens; in addition, as both drugs are available for oral administration, long-term safe use with well-maintained compliance is more likely. In the present study, the incidence of adverse reactions was remarkably low; as expected, the therapeutic regimen was well-tolerated even in elderly patients over the age of 70 years, with

remarkable drug effects. In particular, the prolonged duration of SD of ≥1 year observed in 20% of the treated patients may be said to exemplify the concept of metronomic chemotherapy. Future incorporation of newer molecular-targeting therapeutic agents, besides expanding the available spectrum of therapeutic options, will undoubtedly allow more effective therapy to be individualized according to the patient's background characteristics, taking into account the biology of the breast cancer. Research to elucidate the biology of breast cancer and the accumulation of pertinent cases and prospective studies are necessary in the future.

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Functional interaction of fibroblast growth factor-8, bone morphogenetic protein and estrogen receptor in breast cancer cell proliferation

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ABSTRACT

Estrogen is involved in the development and progression of breast cancer. Here we investigated the effect of fibroblast growth factor (FGF)-8 on breast cancer cell proliferation caused by estrogen using human breast cancer MCF-7 cells. MCF-7 cells express estrogen receptor (ER)α, ERβ, FGF receptors, and Smad signaling molecules. Estradiol stimulated MCF-7 cell proliferation in a concentration-responsive manner, whereas BSA-bound estradiol had a weak effect on MCF-7 cell mitosis compared with the effect of free estradiol. It is notable that estrogen-induced cell proliferation was enhanced in the presence of FGF-8 and that the combined effects were reversed in the presence of an FGF-receptor kinase inhibitor or an ER antagonist. It was also revealed that FGF-8 increased the expression levels of ERα, ERβ and aromatase mRNAs, while estradiol reduced the expression levels of ERs, aromatase and steroid sulfatase in MCF-7 cells. FGF-8-induced phosphorylation of FGF receptors was augmented by estradiol, which was reversed by an ER antagonist. FGF-8-induced activation of MAPKs and AKT signaling was also upregulated in the presence of estrogen. On the other hand, FGF-8 suppressed BMP-7 actions that are linked to mitotic inhibition by activating the cell cycle regulator cdc2. FGF-8 was revealed to inhibit BMP receptor actions including Id-1 promoter activity and Smad1/5/8 phosphorylation by suppressing expression of BMP type-II receptors and by increasing expression of inhibitory Smads. Collectively, the results indicate that FGF-8 acts to facilitate cell proliferation by upregulating endogenous estrogenic actions as well as by suppressing BMP receptor signaling in ER-expressing breast cancer cells.

1. Introduction

The involvement of estrogen in the development and progression of breast cancer is well known (Colditz, 1998; Keen and Davidson, 2003; Fortunati et al., 2010). Increased estrogen exposure via a variety of mechanisms is critical for the development of breast cancer, in which the effects of estrogen are mediated via two estrogen receptor (ER) subtypes, ERα and ERβ. Estrogen and ER complex mediate the activation of protooncogenes and oncogenes, nuclear proteins, and other target genes. However, there is no clear explanation regarding the direct effect of estrogen in the development of breast cancer.

Recent studies have demonstrated the presence of the transforming growth factor (TGF)-β signaling pathway in mammary cells and its importance in maintaining the growth state of these cells. There have been several studies showing the expression of some TGF-β superfamily proteins, such as bone morphogenetic protein (BMP)-2, -6 and -7, in breast cancer cells (Ye et al., 2009; Alarmo and Kallioniemi, 2010), and their possible roles in breast cancer development and in bone metastasis have been suggested. BMPs were originally identified as active components in bone extracts capable of inducing bone formation at ectopic sites. Recently, a variety of physiological BMP actions in endocrine tissues including the ovary, pituitary, thyroid, and adrenal have been clarified (Shimasaki et al., 2004; Otsuka, 2010; Otsuka et al., 2011). Attention has been paid to BMPs for their possible link with

Abbreviations: ActRI and ActRII, activin type-I and -II receptor; ALK, activin receptor-like kinase; Arom, aromatase; BMP, bone morphogenetic protein; BMPRI and BMPRII, BMP type-I and -II receptor; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; MAPK, mitogen-activated protein kinase; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; TGF-β, transforming growth factor-β.

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tumorigenesis, considering the actions of BMPs as regulators of cell proliferation and differentiation. The involvement of BMP-Smad activation in the progression and dedifferentiation of ER-positive breast cancer has also recently been reported (Helms et al., 2005). In this regard, we have reported that BMPs have inhibitory effects on estrogen-induced mitosis of MCF-7 breast cancer cells by inhibiting MAPK pathways and estrogenic enzyme expression (Takahashi et al., 2008). In that study, we found that BMP-6 and -7 antagonize estrogen-induced breast cancer cell proliferation through inhibiting p38 phosphorylation as well as estrogenic enzyme expression. On the other hand, estrogen altered the Smad signaling for BMP-2 and -4 by downregulating specific BMP receptor expression in breast cancer cells. These results suggest the existence of a functional crosstalk between the BMP system and ER actions in breast cancer cells.

Since the first FGF molecule was discovered as a mitogen for cultured fibroblasts, at least 23 distinct FGFs have been identified. FGFs play a critical role in morphogenesis by regulating cell proliferation, differentiation and cell migration during gastrulation and early differentiation of the brain, cranium, pharynx, heart, kidneys and limbs (Heikinheimo et al., 1994; Meyers et al., 1998; Sun et al., 1999; Eswarakumar et al., 2005). During embryonic development, FGF-8 is widely expressed in a temporally and spatially regulated manner (Heikinheimo et al., 1994; Crossley and Martin, 1995). In adult tissues, FGF-8 is expressed at low levels in limited tissues such as ovary and testis (Mattila and Harkonen, 2007); however, human breast, prostate and ovarian tumors can express FGF-8 (Dorkin et al., 1999; Marsh et al., 1999). The mechanism by which FGF-8 is expressed in hormone-responsive tissues including the breast and prostate has yet to be elucidated.

FGF-8 was originally cloned and characterized from androgen-dependent mouse mammary carcinoma cells (Tanaka et al., 1992). FGF-8 has been shown to induce cancer cell proliferation and tumor growth in cell culture (Tanaka et al., 1995; Mattila et al., 2001; Ruohola et al., 2001) and transgenic animal models (Daphna-Iken et al., 1998). Since overexpression of FGF-8 to breast cancer cells provides increased growth potential *in vitro* and *in vivo*, FGF-8 has also been categorized as an oncogene having transforming ability (Mattila and Harkonen, 2007).

During development, there are several organs in which BMP and FGF signals cooperate to regulate cell differentiation. During calvarial suture osteogenesis, FGF-2 augments BMP-4 signaling by suppressing the expression of a BMP antagonist, noggin (Warren et al., 2003). FGF-18 facilitates BMP-2 signaling by suppressing noggin mRNA expression in chondrogenesis (Reinhold et al., 2004). Other examples of cooperation between BMP and FGF signaling have been reported in the nervous system and in ectopic bone formation (Hayashi et al., 2003; Nakamura et al., 2005; Marchal et al., 2009). On the basis of results showing that FGFs and BMPs regulate cell differentiation cooperatively in a cell/tissue-dependent manner, we attempted to clarify the underlying mechanism of ER-sensitive breast cancer cell proliferation through the interrelationship between FGF-8 and the BMP system.

2. Materials and methods

2.1. Reagents and supplies

Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin solution, 17β-estradiol, BSA-conjugated 17β-estradiol (estradiol-BSA) and ICI-182780 (also called fulvestrant) were purchased from Sigma–Aldrich Co., Ltd. (St. Louis, MO). Recombinant human BMP-7 and mouse FGF-8b were purchased from R&D Systems, Inc. (Minneapolis, MN) and SU5402 was purchased from Calbiochem (Gibbstown, NJ). Plasmids of Id-1-Luc and the expression plasmid for Smad4 (pcDEF3-Flag(N)-Smad4) were kindly provided by Drs. Tetsuro Watabe and Kohei Miyazono, Tokyo University, Japan.

2.2. Breast cancer cell culture

The human breast cancer cell line MCF-7 was from American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin solution at 37 °C under a humid atmosphere of 95% air/5% CO_2. In some experiments, cell numbers were counted by culturing MCF-7 cells in 12-well plates (1×10^5 viable cells) with serum-free DMEM for 24 h. The cells were then washed with phosphate-buffered saline (PBS), trypsinized, and counted using a coulter counter (Beckman Coulter Inc., Fullerton, CA). Changes in cell morphology and cell viability were monitored using an inverted microscope.

2.3. RNA extraction, RT-PCR, and quantitative real-time PCR analysis

To prepare total cellular RNA, MCF-7 cells were cultured in 12-well plates (5×10^5 viable cells) and treated with indicated concentrations of estradiol and growth factors including BMP-7 and FGF-8 in serum-free DMEM. In the indicated experiments, 500 ng of an expression plasmid encoding wild-type Smad4 DNA or an empty vector was transfected using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h, and then cells were treated with BMP-7 and FGF-8 in serum-free conditions. After 24-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA), quantified by measuring absorbance at 260 nm, and stored at −80 °C until assay. The extracted RNA (1.0 μg) was subjected to an RT reaction using the First-Strand cDNA synthesis system® (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 °C for 50 min and at 70 °C for 10 min. Oligonucleotides used for RT-PCR were custom-ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the corresponding genes as follows: ERα, 1393–1413 and 1632–1652 (from GenBank Accession #NM_000125); ERβ, 395–415 and 589–609 (from AB006590); aromatase (Arom), 914–934 and 1235–1256 (from M22246); steroid sulfatase (STS), 513–533 and 693–713 (from NM_000351); Id-1, 218–240 and 357–377 (from NM_012797) and a house-keeping gene, ribosomal protein L19 (RPL19), 401–420 and 571–590 (from NM_000981). PCR primer pairs for ALK-2, -3, -6, activin type-II receptor (ActRII), ActRIIB, BMP type-II receptor (BMPRII), Smad6 and Smad7 were individually selected for regular PCR and real-time PCR as we previously reported (Takahashi et al., 2008). Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of ERα, ERβ, Arom, steroid sulfatase, Id-1, ALK-2, -3, -6, ActRII, ActRIIB, BMPRII, Smad6 and Smad7 mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan) under conditions of annealing at 60–62 °C with 4 mM $MgCl_2$, following the manufacturer's protocol. Accumulated levels of fluorescence for each product were analyzed by the second derivative method after melting-curve analysis (Roche Diagnostic Co.), and then, following assay validation by calculating each amplification efficiency, the expression levels of target genes were quantified on the basis of standard curve analysis for each product. For each transcript, all treatment groups were quantified simultaneously in a single LightCycler run. To correct for differences in RNA quality and quantity between samples, the expression levels of target gene mRNA were normalized by dividing the quantity of the target gene by the quantity of RPL19 in each sample. The raw data of each target

mRNA level (/RPL19) were statistically analyzed as indicated and then shown as fold changes in the figures.

2.4. Thymidine incorporation assay

MCF-7 cells (1×10^4 viable cells) were precultured in 12-well plates with DMEM containing 10% FCS for 24 h. After the preculture medium had been replaced with fresh serum-free medium and indicated combinations of estradiol, estradiol-BSA, BMP-7, FGF-8 and the inhibitors were added to the culture medium. For the experiments using estradiol-BSA, estradiol-BSA free of estradiol was prepared as follows: 4 ml of estradiol-BSA (1.25 mM in estrogen dissolved in 50 mM Tris–HCl, pH 8.5) was added to a centrifugal Amicon Ultra filter unit with an MW cut-off of 5000 (Millipore, Bedford, MA) and centrifuged at 4000*g* until 50 μl of retentate remained. The retentate was washed with Tris buffer and the final volume was adjusted to 5 ml (1 mM). After 24-h culture, 0.5 μCi/ml [*methyl*-^{3}H] thymidine (Amersham Pharmacia, Piscataway, NJ) was added and incubated for 3 h at 37 °C. The incorporated thymidine was detected as we previously reported (Takahashi et al., 2008). Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 60 min at 4 °C, and solubilized in 0.5 M NaOH, and radioactivity was determined with a liquid scintillation counter (TRI-CARB 2300TR, Packard Co., Meriden, CT).

2.5. Western immunoblot analysis

Cells (1×10^5 viable cells) were precultured in 12-well plates in DMEM containing 10% FCS for 24 h. After preculture, the medium was replaced with serum-free fresh medium, and then indicated concentrations of estradiol, growth factors including FGF-8 and BMP-7 and the inhibitors were added to the culture medium. After stimulation with hormones or growth factors for indicated periods, cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM sodium fluoride, 2% sodium dodecyl sulfate, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS–PAGE/immunoblotting analysis using anti-FGFR-1 (Flg), -2 (Bek), -3 and -4 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-FGFR antibody (Cell Signaling Technology, Inc., Beverly, MA), anti-phospho- and anti-total-extracellular signal-regulated kinase (ERK) 1/2 MAPK antibody (Cell Signaling Technology, Inc.), anti-phospho- and anti-total-p38 MAPK antibody (Cell Signaling Technology, Inc.), anti-phospho- and anti-total-stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) MAPK antibody (Cell Signaling Technology, Inc.), anti-phospho- and anti-total-AKT antibody, anti-phospho-PKC (pan) antibody, anti-phospho-Smad1/5/8 (pSmad1/5/8) antibody (Cell Signaling Technology, Inc.), anti-phospho-Rb, phoshpho-cdc2, phospho-p53 antibodies (Cell Signaling Technology, Inc.), and anti-actin antibody (Sigma–Aldrich Co., Ltd.). The relative integrated density of each protein band was digitized by NIH image J 1.34s.

2.6. Transient transfection and luciferase assay

Cells (1×10^4 viable cells) were precultured in 12-well plates in DMEM with 10% FCS for 24 h. The cells were then transiently transfected with 500 ng of Id-1-Luc reporter plasmid and 50 ng of cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE 6 (Roche Molecular Biochemicals) for 24 h. The cells were then treated with BMP-7 in combination with FGF-8 in serum-free fresh medium for 24 h. The cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase activity and β-galactosidase (β-gal) activity of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan) as we previously reported (Takahashi et al., 2008). The data were shown as the ratio of luciferase to β-gal activity.

2.7. Statistical analysis

All results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. All the data were subjected to ANOVA with Tukey–Kramer's post hoc test or unpaired *t*-test, when appropriate, to determine differences. *P* values < 0.05 were accepted as statistically significant.

3. Results

Interaction of estrogen and FGF-8 actions were firstly examined in MCF-7 cell proliferation. As shown in the thymidine incorporation assay, estradiol stimulated MCF-7 mitosis in a concentration-responsive manner, while BSA-bound estradiol, which is unable to bind to intracellular estrogen receptors (ERs), had a weak effect on MCF-7 cell mitosis compared with the effects of free estradiol (Fig. 1A). FGF-8 also showed increasing effects on MCF-7 cell mitosis (Fig. 1B). Notably, FGF-8 significantly enhanced the effects of estradiol on MCF-7 cell proliferation (Fig. 1C), and this FGF-8 activity was also seen in experiments using BSA-bound estradiol (Fig. 1D). To clarify the combined effects of FGF-8 and estradiol on cell proliferation, effects of an FGF receptor (FGF-R)-dependent protein kinase inhibitor, SU5402, and an ER antagonist, ICI-182780, were examined. As shown in Fig. 1E, ICI-182780 and SU5402 suppressed estrogen- and FGF-8-induced MCF-7 proliferation, respectively. Either SU5402 or ICI-182780 abolished the combined effects elicited by FGF-8 and estradiol on cell proliferation to the basal level (Fig. 1F), suggesting that both signaling of ER and signaling of FGFR are necessary for the enhancement of MCF-7 cell proliferation.

To clarify the mechanism by which FGF-8 augments estrogen actions in MCF-7 cell mitosis, the activities of FGF-8 receptor signaling were examined by Western blots. FGF receptors including FGFR-1, -2, -3 and -4 were clearly expressed in MCF-7 cells and the expression levels were not affected by estrogen treatments (Fig. 2A). Phosphorylation of FGFRs was detected by treatment with FGF-8 but not by treatment with estradiol. It is of note that estradiol significantly enhanced the effects of FGF-8 on FGFR phosphorylation (Fig. 2B). MAPK, which is a major downstream pathway for FGFRs, was also examined by immunoblots. As shown in Fig. 2C, estradiol, and FGF-8 more potently, stimulated ERK, p38 and SAPK/JNK phosphorylation. Importantly, the treatments with FGF-8 in combination with estradiol significantly enhanced ERK, p38 and SAPK/JNK signaling in MCF-7 cells. In addition, AKT phosphorylation was also stimulated by the combination of estradiol and FGF-8, although PKC activation was not clearly detected by these treatments (Fig. 2D). Moreover, treatments with SU5402, which inhibits FGF-R phosphorylation induced by FGF-8 alone, abolished the enhanced phosphorylation of FGF-R elicited by FGF-8 and estradiol (Fig. 2E). On the other hand, the ER antagonist ICI-182780 reversed the enhanced effects to the levels induced by FGF-8 alone (Fig. 2E). These findings suggest that estradiol enhanced the FGF-8-induced activation of FGF-R signaling via ER action in MCF-7 cells.

To determine the mechanism by which FGF-8 enhances the effects of estrogen in MCF-7 cells, the expression levels of ERs and estrogenic enzymes were examined. As shown in Fig. 3, estradiol decreased the expression levels of ERα, STS and Arom, which might be involved in the autoregulation of ER actions in MCF-7 cells. Importantly, the expression levels of ERα and ERβ were significantly upregulated by FGF-8 even at a lower concentration of 10 ng/ml. Arom expression was also increased in MCF-7 cells

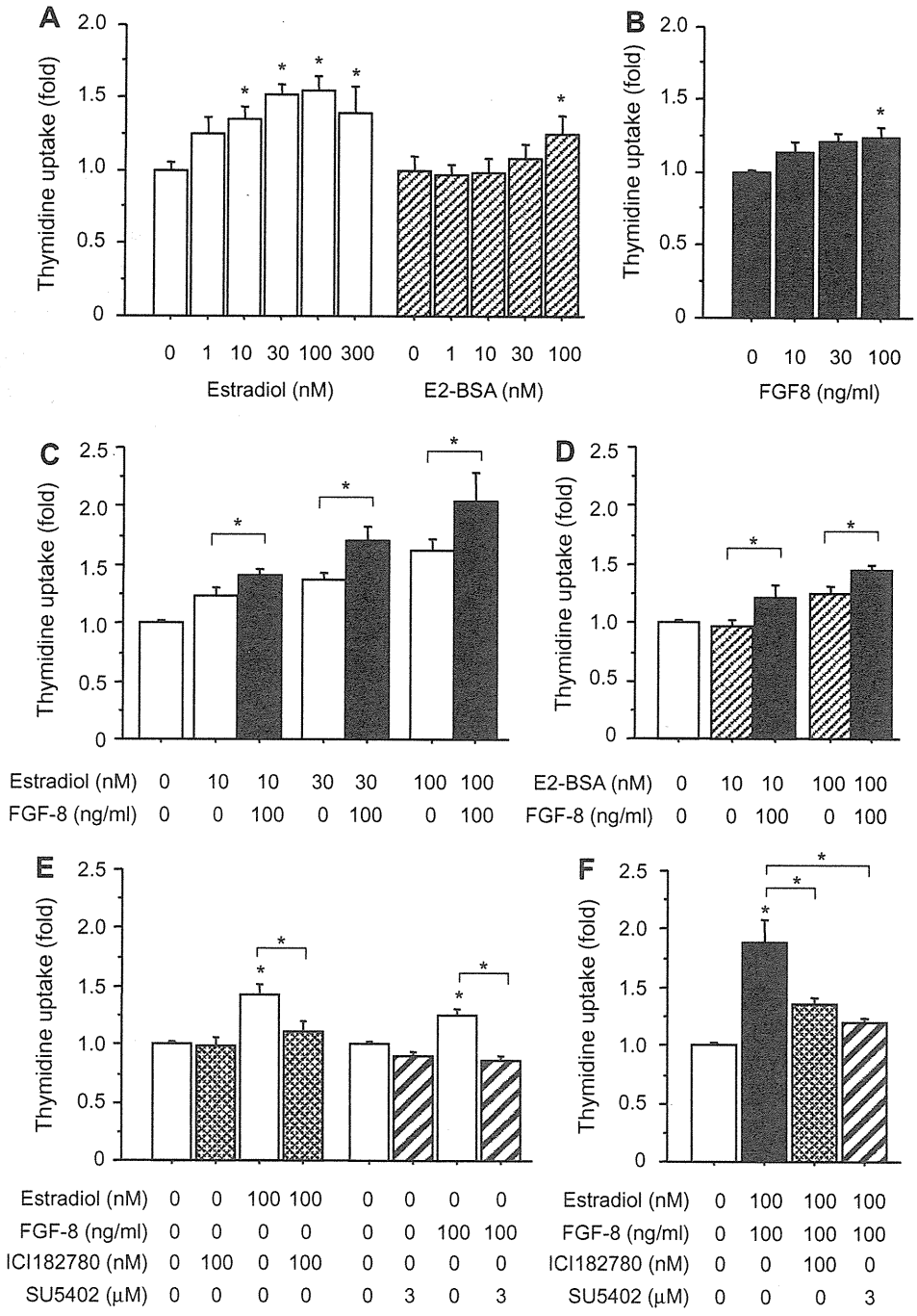

Fig. 1. Effects of estrogen and FGF-8 on MCF-7 cell mitosis. Cells (1×10^4 viable cells) were treated with indicated concentrations of (A) estradiol (E2), E2-BSA, (B) FGF-8 and the combination of (C) E2 and FGF-8, (D) E2-BSA and FGF-8, (E) E2 or FGF-8 and their inhibitors, or (F) E2, FGF-8 and the inhibitors for 24 h in serum-free conditions, and then thymidine uptake assay was performed. Results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey–Kramer's post hoc test or unpaired *t*-test. For each result within a panel, $^*P < 0.05$ vs. control or indicated groups in each panel. Estradiol-BSA indicates estradiol conjugated with bovine serum albumin.

treated with FGF-8 (10 ng/ml), while STS expression was not affected by FGF-8 (Fig. 3).

The interaction between BMP-7 and FGF-8 was also examined. As shown in Fig. 4A, BMP-7 concentration-dependently inhibited MCF-7 cell mitosis induced by estradiol. In the presence of FGF-8, estradiol-induced level of thymidine uptake was augmented; however, the inhibitory effects of BMP-7 on estradiol-induced cell mitosis became insignificant. As shown in the percent reduction of thymidine uptake, the BMP-7 actions that inhibit estrogen-induced mitosis were impaired in the presence of FGF-8 (Fig. 4A). To assess the effects of FGF-8 on BMP-7 signaling activity, the intensity of BMP-7-induced Smad1/5/8 phosphorylation was examined (Fig. 4B). BMP-7-induced Smad1/5/8 phosphorylation was not changed by estradiol, whereas it was moderately impaired by treatment with FGF-8. Notably, the combination of FGF-8 and estradiol markedly suppressed BMP-7-induced phosphorylation

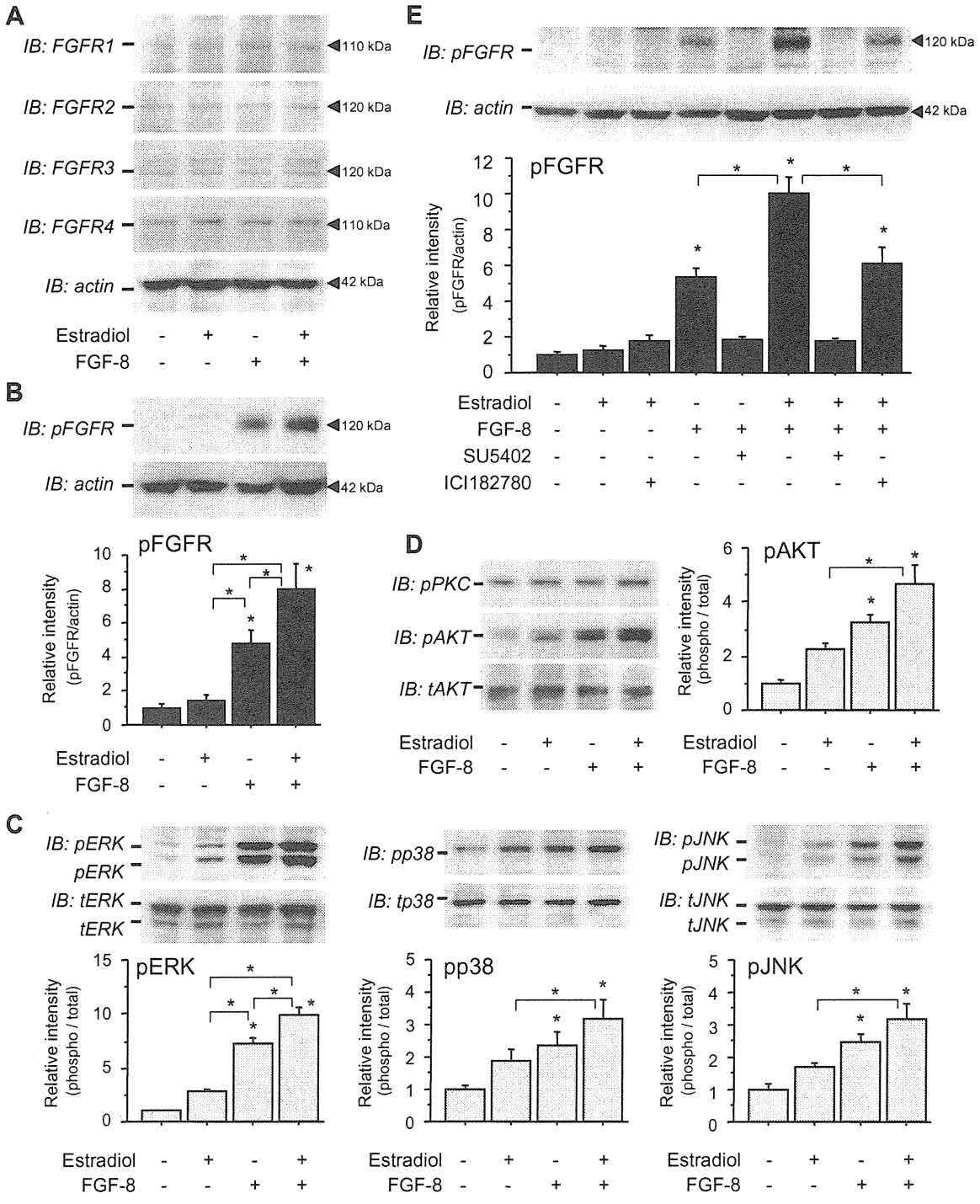

Fig. 2. Expression of FGF receptors and effects of FGF-8 and estrogen on FGF receptor signaling in MCF-7 cells. Cells (1×10^5 viable cells) were cultured with FGF-8 (100 ng/ml) in the presence or absence of estradiol (100 nm) for 24 h, and then cells were lysed and subjected to SDS–PAGE/immunoblotting (IB) analysis using (A) anti-FGFR-1 (Flg), -2 (Bek), -3, -4 and anti-actin antibodies. After preculture, cells (1×10^5 viable cells) were stimulated with FGF-8 (100 ng/ml) in combination with estradiol (100 nm) for 15 min, and then cells were lysed and subjected to IB analysis using (B) anti-phospho-FGFR (pFGFR), (C) anti-phospho- and anti-total-ERK1/2 (pERK/tERK), anti-phospho- and anti-total-p38 (pp38/tp38), anti-phospho- and anti-total-SAPK/JNK (pJNK/tJNK), (D) anti-phospho-PKC (pPKC), and anti-phospho- and anti-total-AKT (pAKT/tAKT) antibodies. E) After preculture with the FGF-R inhibitor SU5402 (3 μM) or the ER antagonist ICI-182780 (100 nm), cells (1×10^5 viable cells) were stimulated with FGF-8 (100 ng/ml) in combination with estradiol (100 nm) for 15 min, and then cells were lysed and subjected to IB analysis using anti-phospho-FGFR (pFGFR). The results shown are representative of those obtained from three independent experiments. In panels (B–E), the relative integrated density of each protein band was digitized and phospho-protein densities were normalized by total-protein or actin levels in each sample. Results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey–Kramer's post hoc test. For each result within a panel, $^*P < 0.05$ vs. control or indicated groups in each panel.

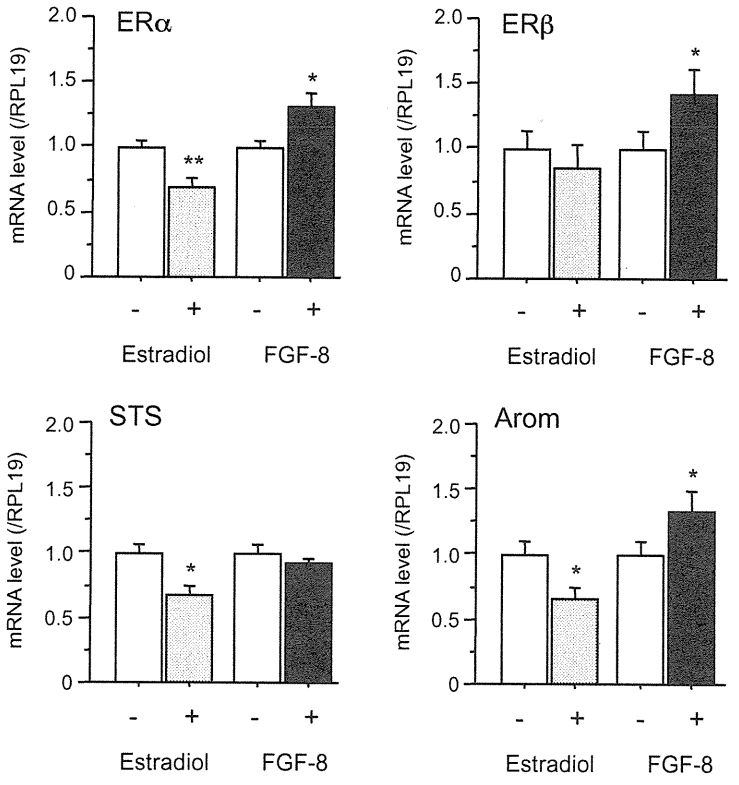

Fig. 3. Effects of FGF-8 on estrogen receptor (ER), steroid sulfatase (STS) and aromatase (Arom) mRNA levels in MCF-7 cells. After preculture, cells were treated with FGF-8 (10 ng/ml) and estradiol (100 nm) for 24 h. Total cellular RNA was extracted and ERα, ERβ, STS, Arom mRNA levels were examined by quantitative real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by unpaired *t*-test. For each result within a panel, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group in each panel.

of Smad1/5/8. Cell cycle checkpoints were also assessed by immunoblots. Among the key cell cycle regulators tested, including Rb (a G1/S regulator), cdc2 (a G2/M regulator) and p53 (a regulator for G2/M and G1/S transition), the activation of cdc2 was induced by BMP-7, suggesting inhibitory actions of BMP-7 on G2 to M phase transition (Fig. 4B).

To clarify the effect of FGF-8 on BMP-7 signaling in MCF-7 cells, transcriptional activity of a BMP target gene, Id-1, was examined (Korchynskyi and ten Dijke, 2002). BMP-7 induced transcriptional activity of Id-1 and this effect was reversed by FGF-8 treatment (Fig. 5A). BMP-7 also increased Id-1 mRNA levels in 12- and 24-h cultures of MCF-7 cells, the changes being stable in 24-h culture conditions (Fig. 5B). Moreover, in accordance with the results shown in Fig. 4B, the inhibitory effect of FGF-8 on BMP-7-induced Id-1 transcription was enhanced in combination with estradiol (Fig. 5C), suggesting that FGF-8 augments estrogen actions with counteracting BMP signaling in MCF-7 cells.

To further explore the mechanism by which FGF-8 inhibits BMP-Smad signaling in MCF-7 cells, changes in the mRNA expression of BMP receptors and Smads were assessed (Fig. 6). As shown in Fig. 6A, the expression levels of BMP type-II receptors, including ActRII, ActRIIB and BMPRII, were decreased by FGF-8 even at a lower concentration (10 ng/ml), while the expression levels of inhibitory Smads, including Smad6 and Smad7, were increased, possibly leading to downregulation of BMP receptor signaling activity in MCF-7 cells. To confirm the correlation of the BMP system in cell proliferation induced by FGF-8 and estradiol, Smad4, a key component of the BMP system, was overexpressed in MCF-7 cells (Fig. 6B). Overexpression of Smad4 (+Smad4) reversed the FGF-8-induced suppression of BMP-7 signaling shown as BMP-induced Id-1 transcription compared with the condition using Mock transfection (−Smad4), suggesting that FGF-8 action is functionally involved in regulation of the BMP system activity in MCF-7 cells.

4. Discussion

ERα expression is frequently detected in breast cancer tissues and the presence of ERα protein has been a standard criterion for adjuvant therapy with antiestrogens that antagonize ER signaling and/or Arom actions (Shupnik, 2007). In contrast, ERβ may act by antagonizing ERα on a specific subset of estrogen-stimulated genes, since the expression of ERβ can be an indicator of therapeutic responses in ERα -positive tumors (Lin et al., 2007). In the present study, estrogen-induced MCF-7 cell proliferation was enhanced in the presence of FGF-8 action. It was also revealed that FGF-8 increased the expression levels of estrogenic property including ERα, ERβ and Arom. Furthermore, FGF-8-induced phosphorylation of FGF receptors, MAPKs and AKT was also upregulated in the presence of estrogen actions. Thus, FGF-8 action is clearly linked to ER-dependent functions in MCF-7 cells (Fig. 7).

Based on the findings that estrogen induced rapid activation of MAPKs, including ERK, p38 and SAPK/JNK pathways, and that BSA-conjugated estradiol, which cannot bind to nuclear ERs (Taguchi et al., 2004), induced modest but significant cell mitosis, nongenomic effects are also involved, at least in part, in the induction of cell proliferation by estrogen. Immunoreactive ER antigen was reportedly detected on the surface of both naturally ER-positive cells and in cells transfected with ER expression

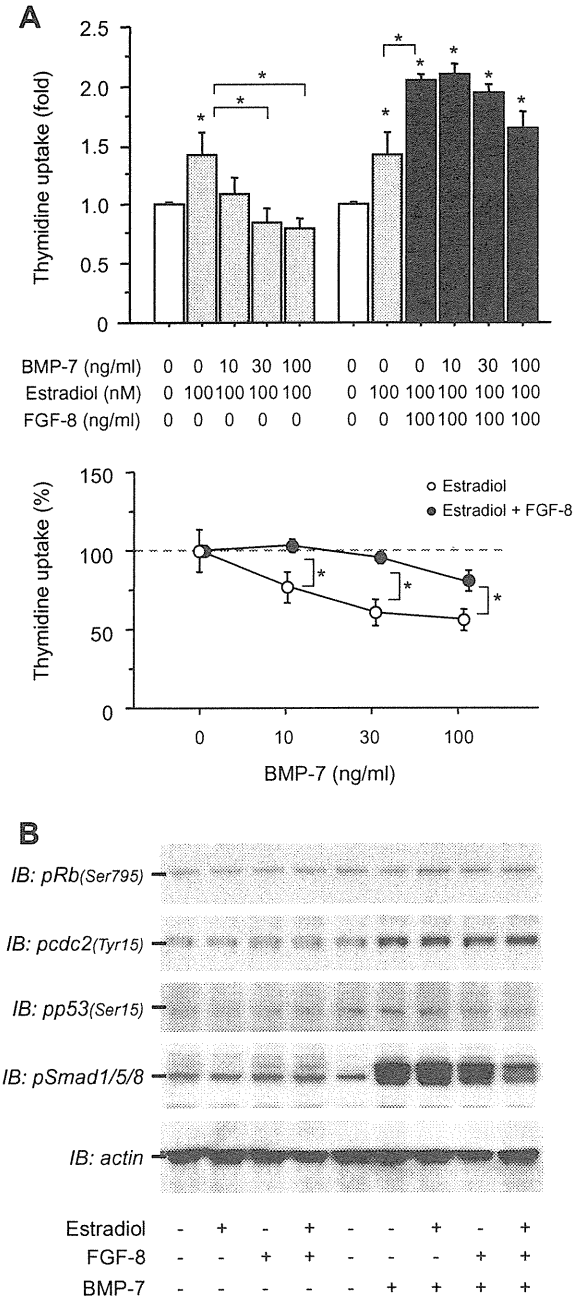

Fig. 4. Effects of BMP-7 and FGF-8 on estrogen-induced cell mitosis and the effects on cell cycle regulators in MCF-7 cells. (A) Cells (1×10^4 viable cells) were treated with indicated concentrations of FGF-8 and BMP-7 in the presence of estradiol (100 nm) in serum-free conditions for 24 h, and then thymidine uptake assay was performed (*upper panel*). The percent changes of thymidine uptake in cells treated with estradiol (100 nm) and BMP-7 (10–100 ng/ml) were compared in the presence or absence of FGF-8 (100 ng/ml) (*lower panel*). Results are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey–Kramer's post hoc test. For each result within a panel, $^*P < 0.05$ vs. control or indicated groups in each panel. (B) Effects of BMP-7, FGF-8 and estrogen on cycle regulator and Smad1/5/8 activation. Cells (1×10^4 viable cells) were precultured for 24 h and stimulated with BMP-7 (100 ng/ml) and FGF-8 (100 ng/ml) in the absence or presence of estradiol (100 nm). After 15-min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-phospho-Rb (pRb), phoshpho-cdc2 (pcdc2), phospho-p53 (pp53), anti-phospho-Smad1/5/8 (pSmad1/5/8) and anti-actin antibodies. The results shown are representative of those obtained from three independent experiments.

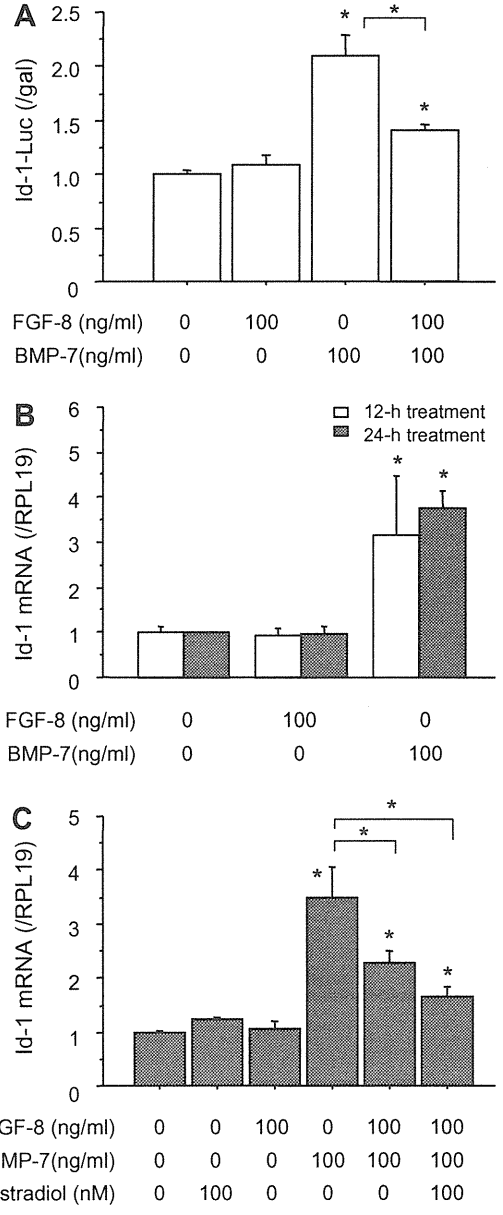

Fig. 5. Effects of FGF-8 on BMP receptor signaling in MCF-7 cells. (A) Cells (1×10^4 viable cells) were transiently transfected with Id-1-Luc reporter plasmid (500 ng) and pCMV-β-gal. After 24-h treatment with BMP-7 (100 ng/ml) and FGF-8 (100 ng/ml), cells were lysed and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β-galactosidase (β-gal) activity. (B, C) After preculture, cells were treated with FGF-8 (100 ng/ml) and BMP-7 in the presence or absence of estradiol (100 nm) in serum-free condition for 12 h or 24 h. Total cellular RNA was extracted and Id-1 mRNA levels were examined by quantitative real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey–Kramer's post hoc test. For each result within a panel, $^*P < 0.05$ vs. control or indicated groups in each panel.

constructs (Watson et al., 2002). ER-transfected cells also resulted in detectable membrane ER, in which estradiol mediates MAPK action, cAMP-PKA activation and the inositol-triphosphate (PI3K) pathway (Razandi et al., 1999). In the present study, FGF-8 upregulated the effects of estradiol not only by regular estrogen but also

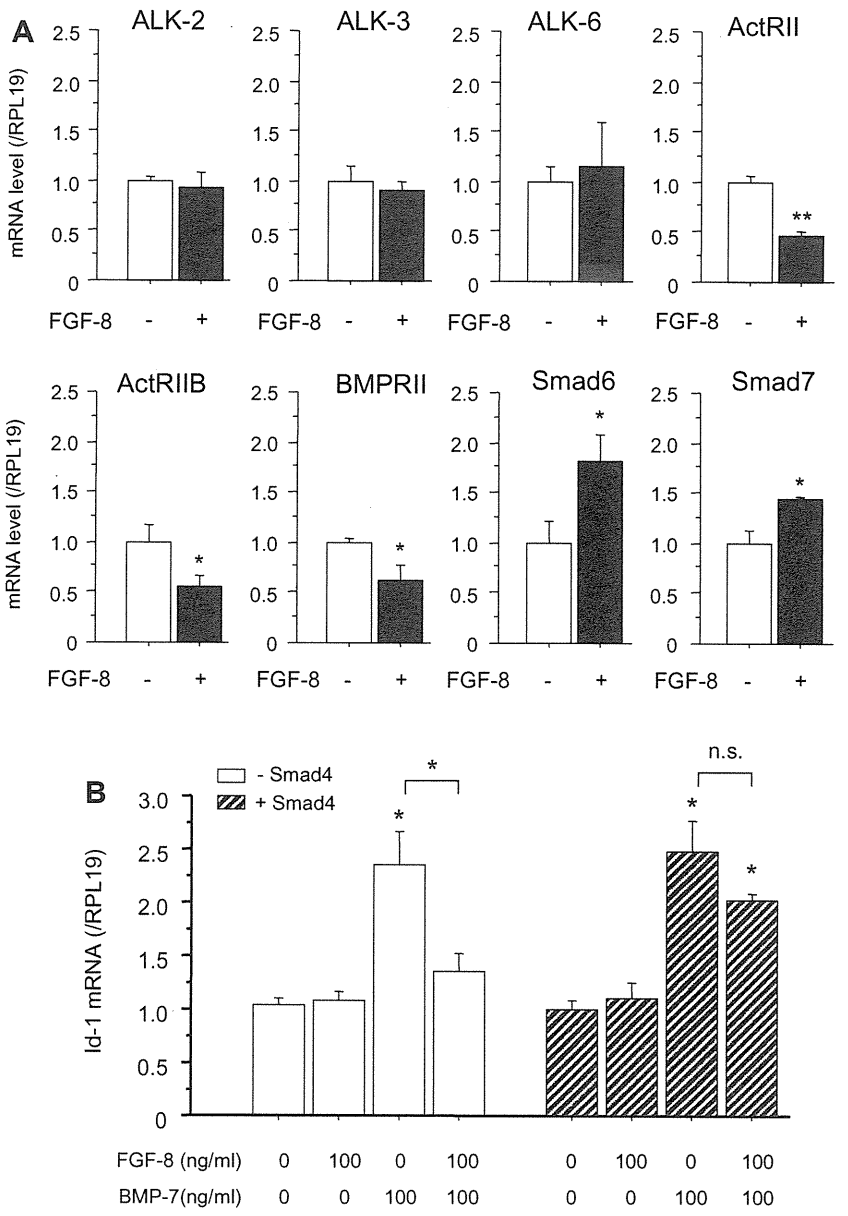

Fig. 6. Effects of estrogen on the expression of BMP system molecules in MCF-7 cells. (A) After preculture, cells were treated with or without FGF-8 (10 ng/ml) for 24 h. Total cellular RNA was extracted and ALK-2, -3, -6, ActRII, ActRIIB, BMPRII, Smad6 and Smad7 mRNA levels were examined by quantitative real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by unpaired *t*-test. For each result within a panel, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control group in each panel. (B) Cells were transfected with 500 ng of an expression plasmid encoding wild-type Smad4 (+Smad4) or an empty vector (−Smad4) and then treated with indicated concentrations of BMP-7 and FGF-8 in serum-free conditions for 24 h. Total cellular RNA was extracted and Id-1 mRNA levels were examined by quantitative real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results in all panels are shown as means ± SEM of data from at least three separate experiments, each performed with triplicate samples. The results were analyzed by ANOVA with Tukey–Kramer's post hoc test. For each result within a panel, $^{*}P < 0.05$ vs. control or indicated groups in each panel; n.s. indicates not significant.

by membrane-impermeable estrogen, suggesting augmentation of ER expression on the cell surface in addition to genomic ERs.

Breast cancer tissues express all enzymes required for local biosynthesis of estrogen from circulating precursors (Foster, 2008). Two principal pathways are implicated in the final steps of estradiol formation in breast cancer tissues: the Arom pathway, which transforms androgens into estrogens, and the STS pathway, which converts estrone sulfate into estrone. The final step of estrogen steroidogenesis is conversion of weak estrone to biologically active estradiol by 17β-hydroxysteroid dehydrogenase type 1. Therefore, therapeutic targets for breast cancers include not only the binding of estrogen to ERs but also the activity of estrogenic enzymes in tumor tissues (Sasano et al., 2006; Subramanian et al., 2007). In our earlier study (Takahashi et al., 2008), BMP-7 efficaciously suppressed STS expression in MCF-7 cells at mRNA and protein levels. Given that STS inhibitors are effective for inhibiting MCF-7 cell proliferation induced by estrone sulfate (Selcer et al., 1997), the effects of BMP-7 on STS expression may also contribute to the inhibition of estrogen-induced breast cancer cell proliferation. In contrast to BMP actions, FGF-8 increased Arom expression in MCF-7 cells, sug-

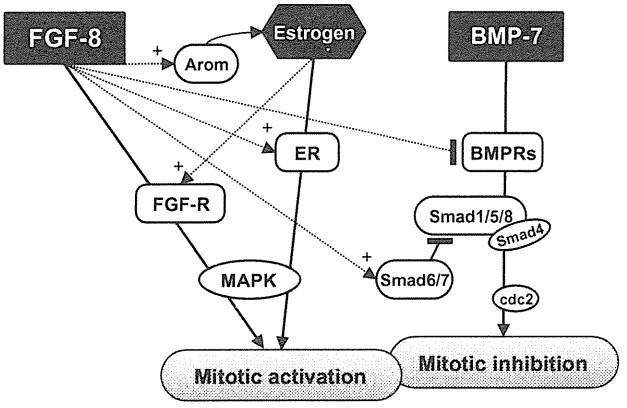

Fig. 7. Interaction of FGF-8, estrogen and BMP activities in breast cancer cell proliferation. Estrogen-induced proliferation is enhanced in the presence of FGF-8. FGF-8 increases the expression levels of ER and aromatase. FGF-8-induced phosphorylation of FGF receptor and activities of MAPKs and AKT signaling are upregulated by estrogen. On the other hand, FGF-8 suppresses BMP-7 actions that are linked to mitotic inhibition by activating the cell cycle regulator cdc2. FGF-8 inhibits BMP receptor-Smad1/5/8 signal activity through suppressing expression of BMP type-II receptors and increasing expression of inhibitory Smad6/7 in MCF-7 cells. Thus, FGF-8 acts to facilitate cell proliferation by enhancing endogenous estrogenic actions as well as by suppressing BMP receptor signaling in ER-positive breast cancer cells.

gesting that the upregulatory effect of the estrogenic machinery of FGF-8, in addition to the expression of ERs, leads to mitogenetic ability of MCF-7 cells.

FGF-8 has been shown to act as an autocrine factor by stimulating proliferation of steroid-sensitive breast and prostate cancer cells (Mattila and Harkonen, 2007). In adult tissues, FGF-8 is expressed in the kidney, breast, prostate and testis in humans, whereas FGF-8 is detected in the heart, brain, lung, kidney, testis, prostate and ovary in rats (Mattila and Harkonen, 2007). Regardless of the species differences, FGF-8 is predominantly detected in steroid-responsive tissues, including reproductive and genitourinary tissues. Hormone dependency of FGF-8 expression in mammary carcinoma cells can be partly explained by the existence of steroid hormone-sensitive promoter elements of mouse mammary tumor virus sequences integrated close to the FGF-8 gene (Valve et al., 1998). The pattern of FGF-8 expression in steroid-responsive tissues also infers the existence of functional interactions between FGF-8 and steroid-receptor signaling and a possible role of steroids in the tissue-specific expression of FGF-8.

Increased expression of FGF-8 has been reported in human breast cancer tissues, which is possibly linked to the growth and progression of breast cancer (Tanaka et al., 1998; Marsh et al., 1999; Zammit et al., 2002). The expression levels of FGF-8 in human breast cancers do not appear to directly correlate with clinical and pathological factors (Tanaka et al., 2002). FGF-8b, having the highest transforming activity (MacArthur et al., 1995), is the predominant isoform detected in human breast cancer (Marsh et al., 1999). Receptors for FGF-8 are also expressed in breast cancer tissues (Mattila and Harkonen, 2007). Enhanced expression of FGFR-1 (Theillet et al., 1993; Yoshimura et al., 1998), -2 (Adnane et al., 1991) and -4 (Jaakkola et al., 1993; Penault-Llorca et al., 1995) have been reported in human breast cancer tissues. In the present study, FGF-8 receptors including FGFR-1 to -4 were expressed and the protein levels were not significantly altered under the condition of treatment with estrogen and FGF-8 in MCF-7 cells.

In the present study, it was also revealed that FGF-8 suppressed BMP-7 actions that are linked to mitotic inhibition by activating the cell cycle regulator cdc2. As a mechanism of the BMP-FGF interaction, FGF-8 was revealed to inhibit BMP receptor signaling activities including Id-1 transcription and Smad1/5/8 phosphorylation through suppressing expression of BMP type-II receptors and increasing expression of inhibitory Smad6/7 in MCF-7 cells (Fig. 7). In addition to the interaction of FGF and BMP in the embryonic phase, there are several functional interplays between BMP and the FGF system in adult tissues. For instance, interaction between BMP and FGF-8 is critical for normal ovarian follicle growth. We have also reported that FGF-8 regulates ovarian steroidogenesis by activating MAPK pathways. Namely, gonadotropin-induced ERK signaling is upregulated by FGF-8 in granulosa cells, which is functionally linked to suppression of estradiol production (Miyoshi et al., 2010). Considering that FGF-8 cooperates with BMP-15, which is a key molecule for human fertility (Otsuka et al., 2011), to promote glycolysis in cumulus cells in mice (Sugiura et al., 2007), the interaction between FGF-8 and BMP receptor signaling is likely to play a key role in regulation of steroidogenesis in the adult ovary.

With regard to the BMP system, several preferential combinations of BMP ligands and receptors have been recognized to date (Shimasaki et al., 2004). BMP-6 and -7 most readily bind to ALK-2 and/or ALK-6. ActRII acts as a receptor not only for activins but also for BMP-6 and -7, while BMPRII binds exclusively to BMP-2, -4, -6, -7 and -15. BMPRII has been shown to play a key role in breast cancer cell proliferation based on results of experiments using dominant-negative BMPRII constructs (Pouliot et al., 2003). Expression of ALK-6 has also been proposed as a major hallmark of progression and prognosis of ER-positive breast cancer (Helms et al., 2005). In this regard, it has also been reported that inhibition of BMP-2-induced Smad signaling by estrogen is due to direct physical interaction between Smads and ERs in MCF-7 cells (Yamamoto et al., 2002). In our earlier study, a functional interrelationship between estrogen actions and BMPs was uncovered in the human breast cancer cell line MCF-7 (Takahashi et al., 2008). Estradiol specifically decreased ALK-3, -6, ActRII and ActRIIB (Takahashi et al., 2008). The expression pattern of BMP receptors in estrogen-primed MCF-7 cells was consistent with the inhibitory effects of BMP ligands on MAPK phosphorylation and estrogen-induced cell proliferation.

In the present study, it was also revealed that BMP-7 inactivated cdc2 by phosphorylation at the position of Tyr15. Cdc2 is phosphorylated at Thr161 to be active and this process is catalyzed in animal cells by CDK-activating kinase (Stark and Taylor, 2006). In mammalian cells, accumulating cdc2/cyclin B complex is kept inactive by two inhibitory phosphorylations on the cdc2 subunit at Tyr15, catalyzed by Wee1, and at Thr14, catalyzed by Myt1. Namely, BMP-7 inactivates a key regulator of cell cycle cdc2 by phosphorylation, leading to the inhibition of G2 to M phase transition of MCF-7 cells. FGF-8 exhibited preferential suppression of all BMP type-II receptors including ActRII, ActRIIB and BMPRII, while FGF-8 increased the expression of inhibitory Smad6 and Smad7. It is intriguing that estrogen and FGF-8, both of which are mitogenic factors in breast cancer cells, act to inhibit the expression of BMP receptor signaling, which plays a key role in the suppression of MCF-7 cell mitosis.

A recent study has shown that FGF-8 increased expression levels of cyclin D1 and Ki67, leading to the promotion of cell cycle progression via ERK and AKT pathways in MCF-7 cells (Nilsson et al., 2010). Moreover, FGF-8 has been reported to have the capacity to act as a survival factor against apoptotic agents via the AKT pathway in mouse breast cancer S115 cells (Nilsson et al., 2010). In the present study, phosphorylation of Rb or p58, a G1/S regulator and a regulator for G2/M and G1/S, respectively, was not detected in MCF-7 cells during the short period of exposure to FGF-8 in combination with estrogen. However, a study using mouse S115 cells suggested that FGF-8-induced cyclin D1 is linked to Rb phosphorylation by formation of active cyclin D1-CDK4/6

complexes (Nilsson et al., 2010), which may possibly allow cell cycle progression through the G1-restriction point, leading to S phase progression. Further study is needed to elucidate the molecular mechanism by which FGF-8 stimulates estrogen-induced cell mitosis and cell cycle progression through the inhibition of BMP receptor signaling.

Collectively, the results indicate that FGF-8 acts to facilitate cell proliferation by enhancing endogenous estrogenic actions as well as by suppressing BMP receptor signaling in ER-positive breast cancer cells (Fig. 7). Given the findings that FGF-8 and FGF receptor expression is increased in human breast cancer tissues, suppression of endogenous FGF-8 functions and/or enhancement of the functional BMP system could be a possible therapeutic strategy to inhibit the development of estrogen-responsive breast cancer.

Acknowledgements

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