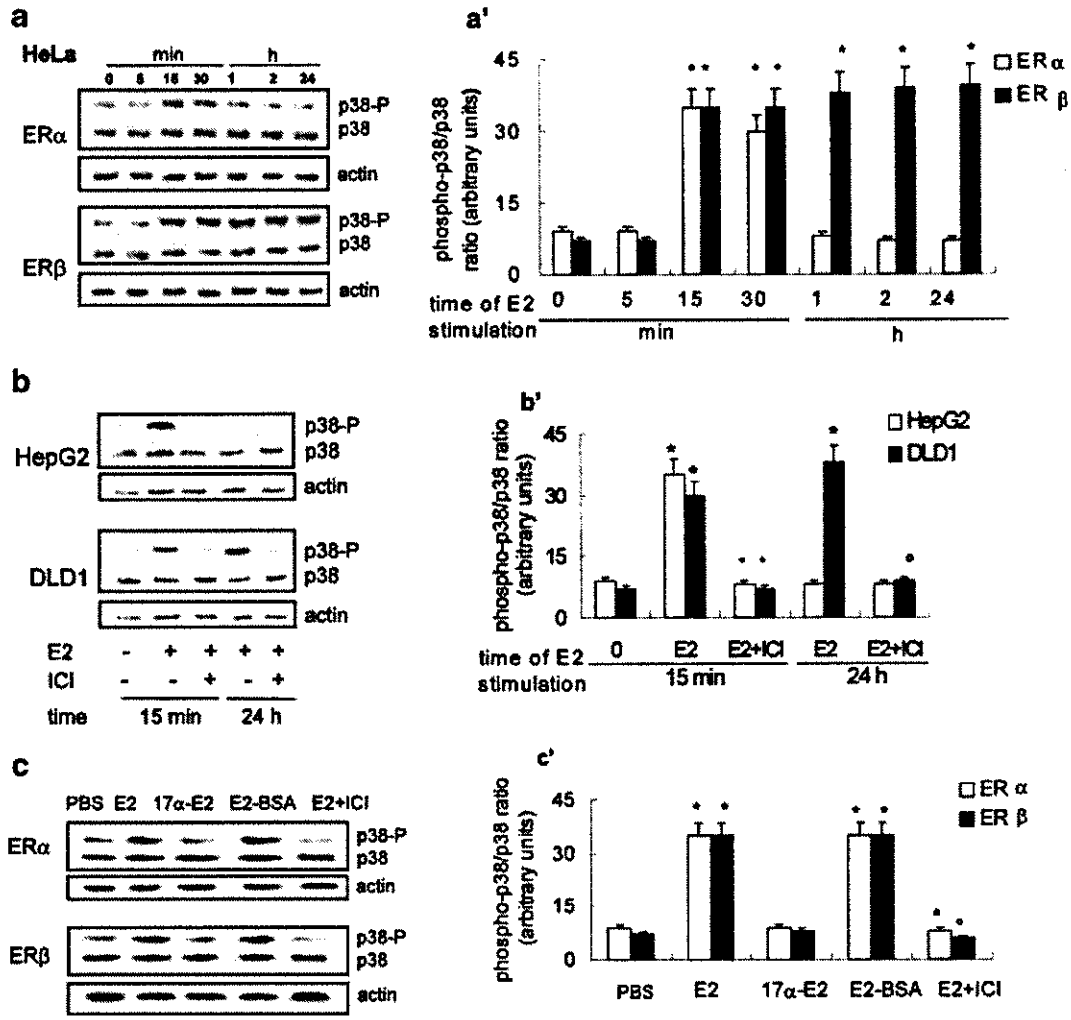

Fig. 4. Effect of E2 on p38/MAPK activation. Time course analysis of p38/MAPK phosphorylation was performed, as described in "Materials and Methods," on untreated (0, –) and E2-treated (10 nM) HeLa cells transfected with human ERα or human ERβ expression vectors (**parts a** and **a'**) or HepG2 or DLD1 cells (**parts b** and **b'**) at the indicated times. The amount of protein levels were normalized by comparison with actin expression. Parts (a) and (b) show the typical blot of three independent experiments; parts (a') and (b') show the data obtained by densitometric analysis, mean values ± SD. $P < 0.001$, calculated with Student's *t*-test, compared with respective un-stimulated (0, –) values (*) or with E2-stimulated values (°). For details see the text. Western blot analysis of p38/MAPK phosphorylation was performed, as described in "Materials and Methods," in un-stimulated (PBS) or 15 min E2- (10 nM) or 17α-estradiol- (17α-E2; 10 nM) or 17β-estradiol-BSA-(E2-BSA; 10 nM) treated HeLa cells transfected with human ERα or human ERβ expression vectors. In some experiments cells were pretreated with ICI 182,780 (ICI) (1 μM) before E2 stimulation. The amount of protein levels were normalized by comparison with actin expression. **Part (c)** shows the typical blot of three independent experiments; **part (c')** shows the data obtained by densitometric analysis, mean values ± SD. $P < 0.001$, calculated with Student's *t*-test, compared with respective un-stimulated control (PBS) values (*) or with E2-stimulated values (°). For details see the text.

both cell cycle progression and apoptotic cascade prevention, the E2–ERβ complex induced the rapid and persistent phosphorylation of p38/MAPK, which in turn, drove cells into the apoptotic cycle.

In the E2-stimulated ERα-containing HepG2 cells, we previously demonstrated that E2 enacted the rapid, non-genomic, and membrane starting signal transduction pathways which, in turn, worked cooperatively to achieve cell proliferation. In particular, E2-induced PKC-α was strongly related to DNA synthesis, but was not involved in cyclin D_1 transcription. On the contrary, E2-induced ERK/MAPK and PI3K/AKT pathways were strongly involved in both DNA synthesis and cyclin D_1 transcription (Marino et al., 2002, 2003). Present results clearly indicate, in well accordance with the literature (Razandi et al., 2000b; Kousteni et al., 2001), that these latter pathways have also a critical role in E2 action as a survival agent. While this work was in progress, Fernando and Wimalasena (2004) demonstrated that the prolonged activity of AKT was required to maintain the BAD phosphorylation decreasing its pro-apoptotic effect. In addition, we demonstrate that the E2-induced rapid activation of PI3K/AKT pathway is necessary to increase the level of the anti-apoptotic protein Bcl-2 and to avoid the cleavage of caspase-3 and the induction of apoptotic cascade. Beside AKT-mediated signaling, E2 can also signal through ERK/MAPK pathway. This pathway precedes and modulates AKT phosphorylation (Marino et al., 2003). In fact, the pre-treatment of HepG2 cells with U 0126 (ERK/MAPK inhibitor) rapidly increased the levels of the tumor-suppressor, PTEN, impairing the E2-induced AKT phosphorylation (Marino et al., 2003).

Work of the last years has established that expression and function of component of death machinery are under control of signaling pathways (see Rapp et al., 2004 and literature therein). ERK/MAPK as well as PI3K/AKT cascades cooperate in cellular protection. ERK/MAPK

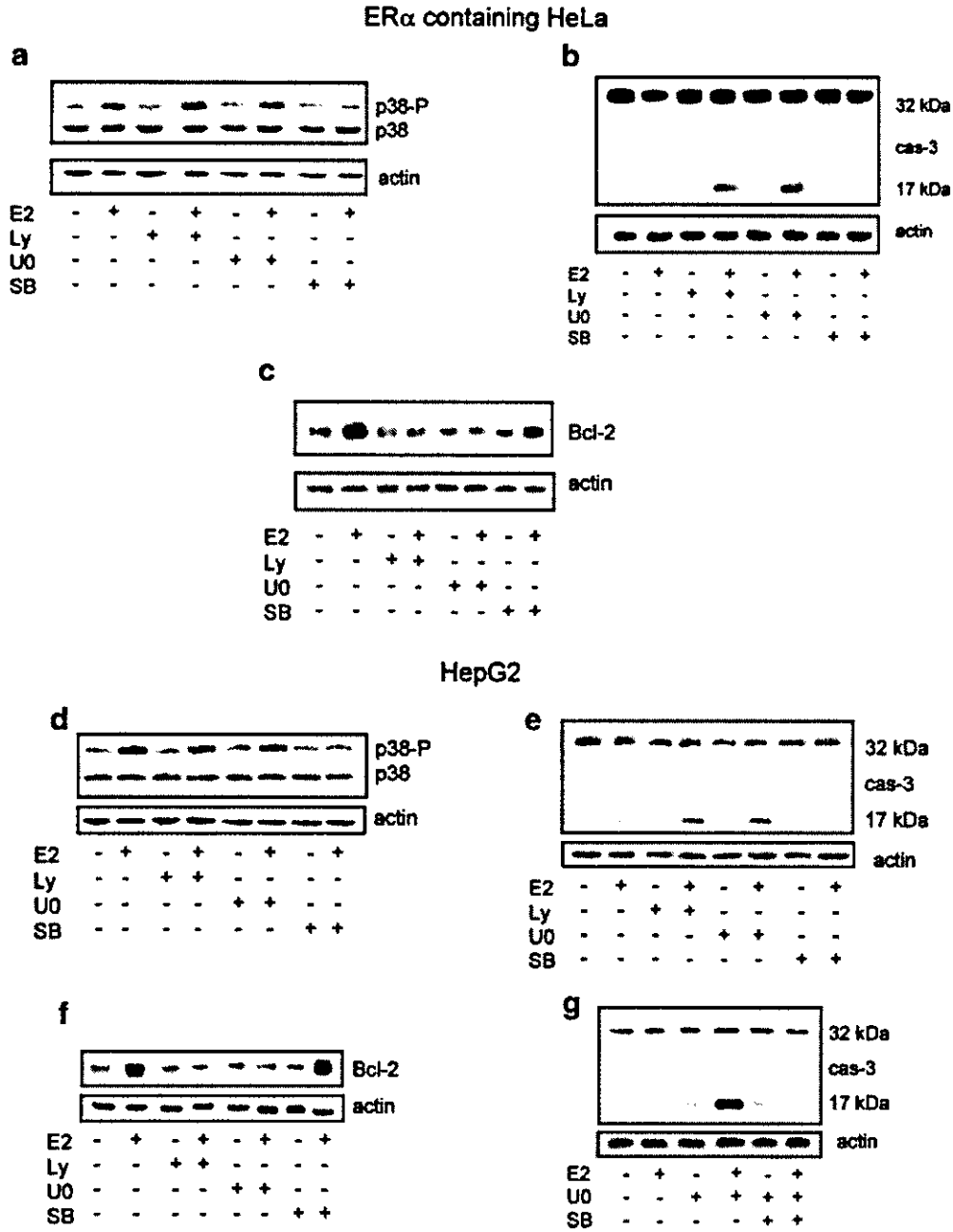

Fig. 5. Cross-talk among E2-induced ERK/MAPK, PI3K/AKT and p38/MAPK activation. Western blot analysis of p38/MAPK (**parts a** and **d**), caspase-3 (**parts b**, **e**, and **g**), and Bcl-2 (**parts c** and **f**) were performed, as described in "Materials and Methods," on un-stimulated (−) or E2-treated (10 nM) (15 min for p38 phosphorylation, 24 h for caspase-3 and Bcl-2 detection) HeLa cells transfected with human ERα expression vector or HepG2 cells. When indicated 10 μM U 0126, Ly 294002 (15 min) or 5 μM SB 203580 (30 min) (ERK/MAPK, PI3K/AKT, and p38/MAPK pathway inhibitors, respectively) were added before E2 administration. The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

control cell survival by targeting Bcl-2 to the mitochondria membranes (Tamura et al., 2004) and together with PI3K/AKT may up-regulate the expression of Bcl-2 (Rapp et al., 2004). Furthermore a direct role of PI3K/AKT in caspase-3 inhibition has been recently reported after polyamine depletion (Zhang et al., 2004). Bcl-2 overexpression, in turn, decreases intracellular Ca^{++} level which can activate p38/MAPK and caspase cascades (Song et al., 2004). Our results, for the first time, show that steroid hormones may regulate this pathway. In fact, the ERK/MAPK and the PI3K/AKT pathways, rapidly activated by the E2–ERα complex, cooperatively enhance the expression of the anti-apoptotic protein (Bcl-2), block the parallel activation of the p38/MAPK, reduce the pro-apoptotic caspase-3 activation, and promote the G1/S transition via the enhancement of cyclin D_1 expression (Marino et al., 2002, 2003; present data).

One of the main findings in this study is the different signal generated by the E2–ERβ complex. There is 96% amino acid identity between the DNA-binding region (C domain) of ERα and ERβ, but in the ligand-binding region (E domain) the homology is only 53% (Kuiper et al., 1998; Matthews and Gustafsson, 2003). As the E domain of ERα is sufficient to elicit non-genomic actions (Marino et al., 2002; Razandi et al., 2002; Acconcia et al.,

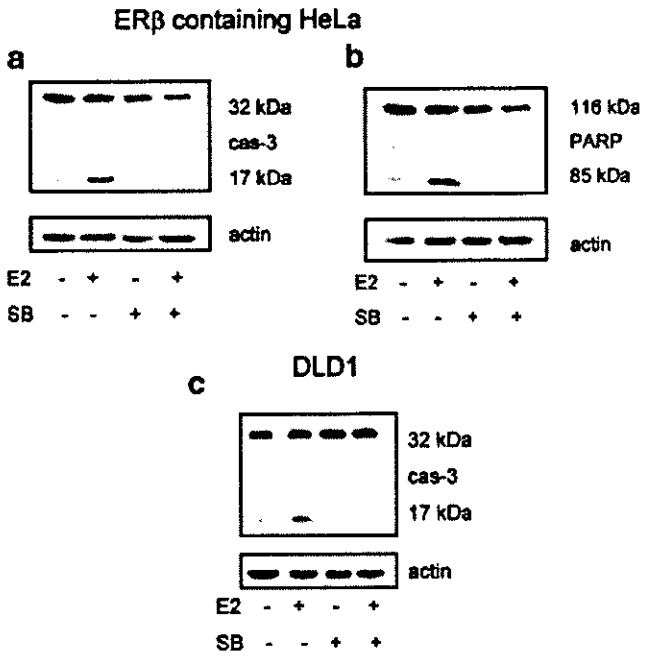

Fig. 6. Involvement of p38/MAPK in pro-apoptotic cascade activation. Western blot analysis of caspase-3 (**parts a** and **c**) and PARP (**part b**) activation were performed, as described in "Materials and Methods," on un-stimulated or 24 h 17β-estradiol-treated (E2; 10 nM) HeLa cells transfected with human ERβ expression vector or in DLD1 cells. When indicated 5 μM SB 203580 (p38/MAPK cascade inhibitor) was added 30 min before E2 administration. The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

2004), it is most likely that different sets of signal transduction proteins may be activated by ERα and ERβ upon E2 binding. Besides these differences the two receptors display a different tissue localization and a different role in proliferation. For example, E2–ERα is a proliferative factor in the uterus, and the uterus of ERβ null mice is hypersensitive to the proliferative action of E2; the co-expression of both ER isoforms is rare during the proliferative phase of mammary gland cells typical of pregnancy, whereas more than 90% of ERβ-expressing mammary gland cells do not proliferate (Weihua et al., 2003); ERβ is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumor's dedifferentiation (Konstantinopoulos et al., 2003); a progressive decline of ERβ expression has been found in multistage mammary carcinogenesis (Roger et al., 2001) and prostate cancer (Horvath et al., 2001). Very recently it has been reported that the induction of ERβ expression reduces the growth of exponentially proliferating breast cancer cells with a parallel decrease in components of the cell cycle associated with proliferation, namely cyclin D_1, cyclin E, Cdc25A, $p45^{Kip2}$ and an increase in the Cdk inhibitor $p27^{Kip1}$ (Matthews and Gustafsson, 2003; Paruthiyil et al., 2004; Strom et al., 2004). Our data amplify these evidences by adding the ability of ERβ isoform to rapidly induce the persistent membrane starting activation of p38/MAPK without any interference on the survival proliferative pathways, thus impairing the cell cycle components activation.

However, we were surprised to find that the E2–ERα complex increased p38/MAPK phosphorylation. Recently, Lee and Bai (2002) reported that in ERα-expressing endometrial cells, E2 activates the p38/MAPK pathway, which in turn mediates the ERα phosphorylation on threonine-311, promoting the receptor nuclear localization and interaction with specific receptor coactivators. In line with this result the E2-induced p38/MAPK phosphorylation plays a multifunctional role in cellular E2-induced effects. As discussed above, the contemporary increase of Bcl-2 levels, mediated by ERK/MAPK and PI3K/AKT pathways, may decrease the Ca^{++} levels impairing the prolonged p38/MAPK activation (Song et al., 2004).

Ample evidence indicates that the p38/MAPK pathway serves an important role in stress and immune response (Han et al., 1994). Furthermore, p38/MAPK pathway has been associated with a significant slowing in cell proliferation (Han et al., 1994; Badger et al., 1996) and with the regulation of the apoptosis (Kang et al., 2003). In particular p38/MAPK can sensitize cells to apoptosis through the positive regulation of Fas/CD-95 and Bax expression which, in turn, activate caspase cascades (Porras et al., 2004). The E2 capacity in activating p38/MAPK has been reported in a few articles and linked to the preservation of form of ERα- and ERβ-containing vascular endothelial cells (Razandi et al., 2000a), or their migration and proliferation (Geraldes et al., 2003), or even their apoptosis (Mori-Abe et al., 2003). Zhang and Shapiro (2000) reported the ability of E2 to induce p38/MAPK phosphorylation and cell apoptosis in a clone of ERα stably transfected HeLa cells (HeLa-ER5), unresponsive to the E2 proliferative stimuli. The reason for these disparities is not clear but could be related to the divergences in the experimental models, colture condition, E2 treatment period, proliferative capacity, or cell line variability. The E2 stimulation of two cell lines containing endogenous (DLD1) or transfected (HeLa) ERβ demonstrates the ability of human ERβ to drive cancer cells to apoptosis via p38/MAPK-cascade.

In conclusion, besides its role as negative modulator of ERα activities, our findings indicate that ERβ directs the anti-proliferative effects of E2 sustaining the tumor suppressor functions of ERβ. Therefore, the expression of ERs could account for the E2-dependent modulation of cell proliferation. In particular, E2 promotes cell survival through ERα-non-genomic signaling and cell death through ERβ-non-genomic signaling. Thus, the E2 opposite effects in cells co-expressing ERα and ERβ could depend on the balance between the signals originated by each isoform. However, the appearance of new and different signals in the presence of either receptors can not be excluded and it is currently under active investigation in our laboratory.

ACKNOWLEDGMENTS

The generous gift of DLD1 cells from Dr. Aldo Cavallini (Biochemistry Laboratory, I.R.C.C.S.S. de Bellis, Scientific Institute for Digestive Diseases, Via della Resistenza, I-70013 Castellana Grotte, Bari, Italy) is acknowledged. The editorial assistance of Mr. Peter De Muro is also acknowledged. This work was supported by grants from FIRB 2001 and 2004 University "Roma Tre" to Maria Marino.

LITERATURE CITED

Acconcia F, Ascenzi P, Fabozzi G, Visca P, Marino M. 2004. S-palmitoylation modulates human estrogen receptor-alpha functions. Biochem Biophys Res Commun 6:878–883.

Ambrosino C, Nebreda AR. 2001. Cell cycle regulation by p38 MAP kinases. Biol Cell 93:47–51.

Badger AM, Bradbeer JN, Votta B, Lee JC, Adams JL, Griswold DE. 1996. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. J Pharmacol Exp Ther 279:1453–1461.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 772:248–254.
Castoria G, Barone MV, Di Domenico M, Bilancio A, Ametrano D, Migliaccio A, Auricchio F. 1999. Non-trascriptional action of oestradiol and progestin triggers DNA synthesis. EMBO J 18:2500–2510.
Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M, Fiorentino R, Varricchio L, Barone MV, Auricchio F. 2001. PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. EMBO J 20:6050–6059.
Cheng J, Lee EJ, Madison LD, Lazennec G. 2004. Expression of estrogen receptor β in prostate carcinoma cells inhibits invasion and proliferation and triggers apoptosis. FEBS Lett 566:169–172.
Couse JF, Korach KS. 1999. Reproductive phenotypes in the estrogen receptor-α knockout mouse. Ann Endocrinol 60:143–148.
Di Leo A, Messa C, Cavallini A, Linsalata M. 2001. Estrogens and colorectal cancer. Curr Drug Targets Immune Endocr Metabol Disord 1:1–12.
Dubal DB, Wilson ME, Wise PM. 1999. Estradiol: A protective and trophic factor in the brain. J Alzheimers Dis 1:265–274.
Fernando RI, Wimalasena J. 2004. Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras dependent non-genomic pathways requiring signaling through ERK and Akt. Mol Biol Cell 15:3266–3284.
Fiorelli G, Picariello L, Martineti V, Tonelli F, Brandi ML. 1999. Functional estrogen receptor β in colon cancer cells. Biochem Biophys Res Commun 261:521–527.
Geraldes P, Sirois MG, Tanguay J-F. 2003. Specific contribution of estrogen receptors on mitogen-activated protein kinase pathways and vascular cell activation. Circulation Res 93:399–405.
Han J, Lee JD, Bibbs L, Ulevitch RJ. 1994. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265:808–811.
Harper SJ, LoGrasso P. 2001. Signalling for survival and death in neurones: The role of stress-activated kinases, JNK and p38. Cell Signal 13:299–310.
Horvath LG, Henshall SM, Lee CS, Head DR, Quinn DI, Makela S, Delprado W, Golovsky D, Brenner PC, O'Neill G, Kooner R, Stricker PD, Grygiel JJ, Gustafsson J-Å, Sutherland RL. 2001. Frequent loss of estrogen receptor-β expression in prostate cancer. Cancer Res 61:5331–5335.
Kang HJ, Soh Y, Kim MS, Lee EJ, Surh YJ, Kim HR, Kim SH, Moon A. 2003. Roles of JNK-1 and p38 in selective induction of apoptosis by capsaicin in Ras-transformed human breast epithelial cells. Int J Cancer 103:475–482.
Konstantinopoulos PA, Kominea A, Vandoros G, Sykiotis GP, Andricopoulos P, Varakis I, Sotiropoulou-Bonikou G, Papavassiliou AG. 2003. Oestrogen receptor beta (ERβ) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. Eur J Cancer 39:1251–1258.
Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Robertson PK, Weinstein RS, Jilka RL, Manolagas SC. 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: Dissociation from transcriptional activity. Cell 104:719–790.
Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J-Å. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139:4252–4263.
Lee H, Bai W. 2002. Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. Mol Cell Biol 22:5835–5845.
Lobenhofer EK, Huper G, Iglehart JD, Marks JR. 2000. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ 11:99–110.
Marino M, Pallottini V, Trentalance A. 1998. Estrogens cause rapid activation of IP_3-PKC-α signal transduction pathway in HepG2 cells. Biochem Biophys Res Commun 245:254–258.
Marino M, Distefano E, Trentalance A, Smith CL. 2001. Estradiol-induced IP_3 mediate the estrogen receptor activity expressed in human cells. Mol Cell Endocrinol 182:19–26.
Marino M, Acconcia F, Bresciani F, Weisz A, Trentalance A. 2002. Distinct non genomic signal transduction pathways controlled by 17β-estradiol regulate DNA synthesis and cyclin D_1 gene transcription in HepG2 cells. Mol Biol Cell 13:3720–3729.
Marino M, Acconcia F, Trentalance A. 2003. Biphasic estradiol-induced AKT-phosphorylation is modulated by PTEN via MAP kinase in HepG2 cells. Mol Biol Cell 14:2583–2591.
Matthews J, Gustafsson J-Å. 2003. Estrogen signalling: A subtle balance between ERα and ERβ. Mol Interventions 3:281–292.
Moon WS, Chang K, Tarnawski AS. 2004. Overexpression of metastatic tumor antigen 1 in hepatocellular carcinoma: Relationship to vascular invasion and estrogen receptor-α. Hum Pathol 35:424–429.
Mori-Abe A, Tsutsumi S, Takahashi K, Toya M, Yoshida M, Du B, Kawagoe J, Nakahara K, Takahashi T, Ohmichi M, Kurachi H. 2003. Estrogen and raloxifene induce apoptosis by activating p38 mitogen-activated protein kinase cascade in synthetic vascular smooth muscle cells. J Endocrinol 178: 417–426.
Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y, Muramatsu M. 1998. Molecular cloning and characterization of human estrogen receptor βcx: A potential inhibitor of estrogen action in human. Nucleic Acids Res 26:3505–3512.
Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. 2004. Estrogen receptor β inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res 64:423–428.
Porras A, Zuluaga S, Black E, Valladares A, Alvarez AM, Ambrosino C, Benito M, Nebreda AR. 2004. p38α mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. Mol Biol Cell 15:922–933.
Rapp UR, Rennefahrt U, Troppmair J. 2004. Bcl-2 proteins: Master switches at the intersection of death signaling and the survival control by Raf kinases. Biochim Biophys Acta 1644:149–158.
Razandi M, Pedram A, Greene GL, Levin ER. 1999. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: Studies of ERα and ERβ expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307–319.
Razandi M, Pedram A, Levin ER. 2000a. Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. Mol Endocrinol 14:1434–1447.
Razandi M, Pedram A, Levin ER. 2000b. Estrogen signals to the preservation of endothelial cell form and function. J Biol Chem 275:38540–38546.
Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. 2002. ERs associate with and regulate the production of caveolin: Implications for signaling and cellular actions. Mol Endocrinol 16:100–115.
Roger P, Sahla ME, Makela S, Gustafsson J-Å, Baldet P, Rochefort H. 2001. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. Cancer Res 61:2537–2541.
Shimada K, Nakamura M, Ispida E, Kishi M, Konishi N. 2003. Roles of p38- and c-jun NH2-terminal kinase-mediated pathways in 2-methoxyestradiol-induced p53 induction and apoptosis. Carcinogenesis 24:1067–1075.
Song RX, Santen RJ. 2003. Apoptotic action of estrogen. Apoptosis 8:55–60.
Song RX, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, Yue W, Wang J, Santen RJ. 2001. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17β-estradiol. J Nat Cancer Institute 93:1714–1723.
Song YS, Park HJ, Kim SY, Lee SH, Yoo HS, Lee HS, Lee MK, Oh KW, Kang SK, Lee SE, Hong JT. 2004. Protective role of Bcl-2 on β-amyloid-induced cell death of differentiated PC12 cells: Reduction of NF-κB and p38 MAP kinase activation. Neurosci Res 49:69–80.
Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson J-Å. 2004. Estrogen receptor beta inhibits 17β-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci USA 101:1566–1571.
Sutherland RL, Reddel RR, Green MD. 1983. Effects of estrogens on cell proliferation and cell cycle kinetics: A hypothesis on the cell cycle effects of antiestrogens. Eur J Cancer Clin Oncol 19:307–318.
Talapatra S, Thompson CB. 2001. Growth factor signaling in cell survival: Implications for cancer treatment. J Pharmacol Exp Ther 298:873–878.
Tamura Y, Simizu S, Osada H. 2004. The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria. FEBS Lett 569:249–255.
Weihua Z, Andersson S, Cheng G, Simpson ER, Warner M, Gustafsson J-Å. 2003. Update on estrogen signaling. FEBS Lett 46:17–24.
Zhang CC, Shapiro DJ. 2000. Activation of the p38 mitogen-activated protein kinase pathway by estrogen or by 4-hydroxytamoxifen is coupled to estrogen receptor-induced apoptosis. J Biol Chem 275:479–486.
Zhang HM, Rao JN, Guo X, Liu L, Zou T, Turner DJ, Wang JY. 2004. Akt kinase activation blocks apoptosis in intestinal epithelial cells by inhibiting caspase-3 after polyamine depletion. J Biol Chem 279:22539–22547.
Zheng J, Ali A, Ramirez VD. 1996. Steroids conjugated to bovine serum albumin as tools to demonstrate specific steroid neuronal membrane binding sites. J Psych Neurosci 21:187–197.

Arch Histol Cytol, 67 (5): 000-000 (2004)

Estrogen receptors and their downstream targets in cancer

Kazuhiro Ikeda[1] and Satoshi Inoue[1, 2]

[1]Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical School, Hidaka; and [2]Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Summary. Estrogen has crucial roles in the proliferation of cancer cells in reproductive organs such as the breast and uterus. Estrogen-stimulated growth requires two estrogen receptors (ERα and ERβ) which are ligand-dependent transcription factors. High expression of ERs is observed in a large population of breast tumors. In addition, the positive expression of ERs correlates with well-differentiated tumors, a favorable prognosis, and responsiveness to an endocrine therapy with anti-estrogen drugs in patients with breast cancer. Transcription activities of ERs can be regulated by interacting proteins such as coactivators and kinases as well as ligand-binding. Moreover, ER isoforms lacking an ability to transactivate are involved in breast cancer. Downstream target genes of ERs have important roles in mediating the estrogen action in breast cancer. We have isolated and characterized several novel estrogen-responsive genes to clarify the molecular mechanism of the estrogen action in target cells. Among these genes, the estrogen-responsive finger protein (Efp) was found to be highly expressed in breast cancer. Efp as a ubiquitin ligase (E3) is involved in the proteasome-dependent degradation of the 14-3-3σ protein, one of cell cycle brakes, this degradation resulting in the promotion of breast cancer growth. A full understanding of the expression and function of ERs and their target genes could shed light on how estrogen stimulates the initiation and promotion of cancer, providing a new approach to diagnose and treat cancer.

Received November 30, 2004

Address for correspondence: Dr. Satoshi Inoue, Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical School, 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, Japan
Phone: +81-42-985-7206, Fax: +81-42-985-7209
E-mail: s_inoue@saitama-med.ac.jp

Background

Estrogen, a sex steroid hormone, exhibits important biological functions in the target tissues such as reproductive organs. Among these tissues, the growth of the mammary gland and uterine endometrium during pregnancy and the menstrual cycle is dependent on estrogen. In addition to proliferative effects on normal cells, estrogen is considered as a stimulant for the initiation and promotion of tumors in these organs. Epidemiological studies show that prolonged exposure to estrogen, i.e. early menarche, late menopause, and estrogen replacement therapy, can be a risk factor in breast and uterine cancers (Rose, 1996; Clemons and Goss, 2001). In vitro experiments indicate that cells derived from breast and uterine tumors are capable of growing in response to estrogen administration (Holinka *et al.*, 1986; Foster *et al.*, 2001). It is reasonable to assume that the stimulatory effects of estrogen on cell proliferation also contribute to malignant tumor growth. Following prolonged exposure to estrogen, an increase in cell proliferation would be expected to cause an increase in spontaneous DNA replication errors. When mutated in target cells of estrogen, it would enhance the replication of clones of cells carrying such genetic errors. It is, therefore, important to understand mechanisms by which estrogen increases cell proliferation in estrogen-associated cancer.

The estrogen-stimulated growth in tumor cells as well as in normal cells requires the estrogen receptor (ER). It has been shown that about two-thirds of human breast tumors express higher concentrations of ERs than normal breast tissues (Early Breast Cancer Trialists' Collaborative Group, 1998). The ER expression status is related to a variety of histologic characteristics of breast cancer. Most tumors with low grades are ER-positive but, in contrast, tumors demonstrating histologic evidence of poor tumor differentiation are frequently ER-negative (Millis, 1980; Fisher *et al.*, 1981). Breast tumors which lack any ER expression often reveal more aggressive phenotypes (Clarke *et al.*,

1994).

Clinically, endocrine therapy with anti-estrogen drugs or aromatase inhibitors is utilized to treat hormone-related cancer (Howell, 2000; Ali and Combos, 2002). It is expected that tamoxifen, an anti-estrogen drug, binds to ER, making it nonfunctional, while aromatase inhibitors reduce estrogen levels. As discussed below, most breast tumors expressing ER are primarily able to respond to tamoxifen. Aromatase inhibitors such as anastrozole and letrozole are especially useful in patients who are or become resistant to tamoxifen. However, a substantial portion of patients with breast cancer eventually acquire resistance against these treatments. In addition, most of the ER-negative breast tumors can not respond to the anti-estrogen drug. Furthermore, several side effects by treatment with tamoxifen and aromatase inhibitors to ER positive cancer such as breast and cancer, have been reported (Wiseman and Adkins, 1998; Buzdar and Hortobagyi, 2000; Howell, 2000; The ATAC Trialists' Group, 2002).

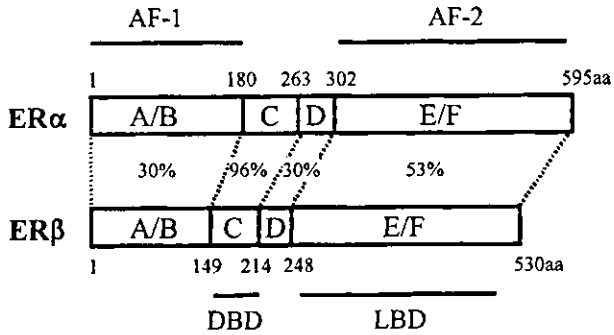

Fig. 1. Schematic representation of human ERα and ERβ. The ERα and ERβ are transcription factors whose activities are regulated by their ligand binding. ERs are members of a nuclear receptor superfamily comprised of six regions (A–F). The ligand-binding domain (LBD) in region E also contains an estrogen-inducible transcription-activating function called AF-2. A constitutively active transcription-activating function (AF-1) is located in the A/B region. Percentages of amino acid identities between the corresponding regions are represented.

It is thus important to uncover the precise mechanism of the estrogen action in breast cancer. In particular, the elucidation of regulatory mechanisms for the expression and function of ERs could provide useful information to predict the responsiveness to endocrine therapy and the prognosis. Moreover, it is important to reveal roles of downstream target genes for ERs, which mediate the effects of estrogen on the proliferation of cancer cells while these genes can be targeted to treat and diagnose estrogen-associated tumors.

Estrogen receptors

As stated above, ER has two subtypes, ERα and ERβ. They belong to the superfamily of nuclear receptors that share similar structures and modes of action (Nuclear Receptors Committee, 1999) (Fig. 1). Namely, estrogen-bound ERs bind as a homodimer or as a heterodimer to an estrogen-responsive element (ERE) with their DNA-binding domain and regulate the transcription of the target genes. ERs contain two independent transcriptional activation functions (AF): the N-terminal A/B domain possesses an autonomous

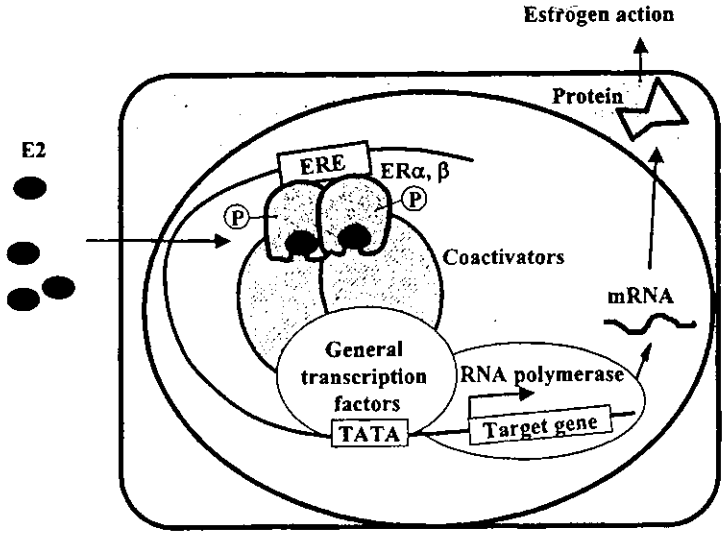

Fig. 2. A model for the regulation of estrogen receptor (ER)-mediated transcription of estrogen-responsive genes. Liganded ERα and ERβ bind as a homodimer or as a heterodimer with an estrogen-responsive element and regulate the target gene transcription. Coactivators are required to mediate ligand-activated transcription by enhancing nuclear receptor transactivation through contacts with the basal transcriptional machinery. Phosphorylation of the ER also modulates the transcription activity.

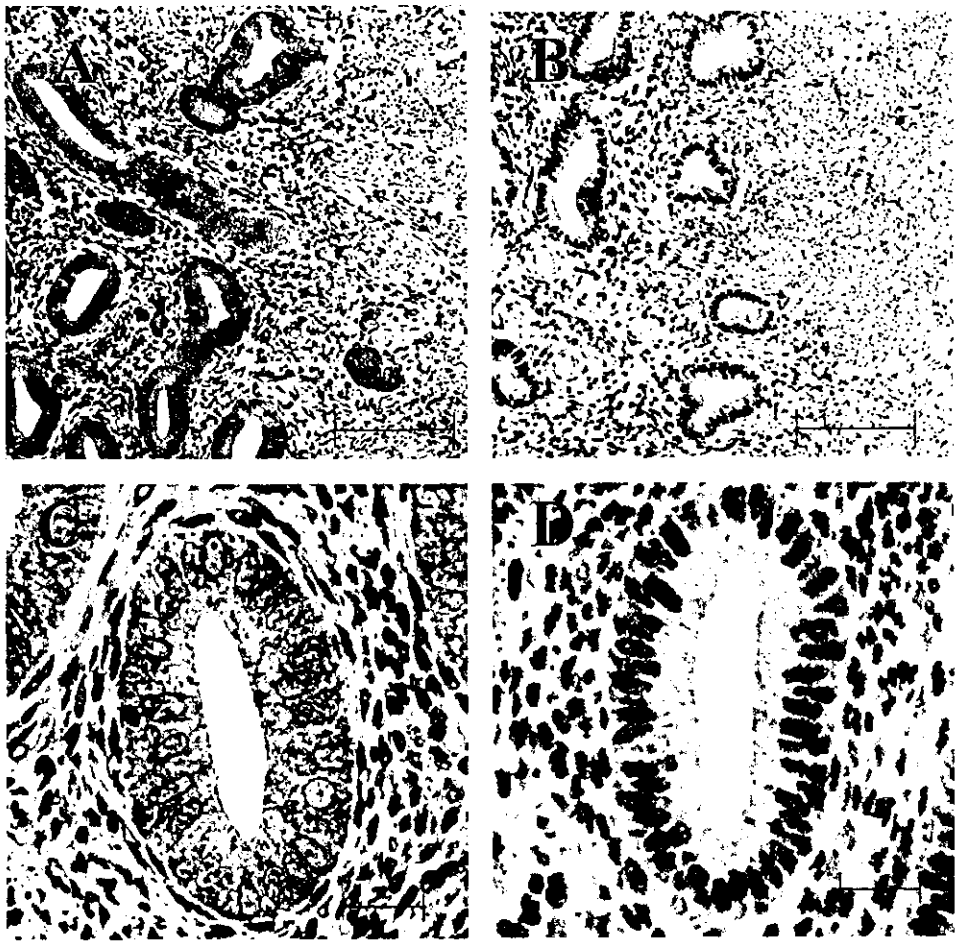

Fig. 3. Immunohistochemical staining of COX7RP (**A** and **C**) and ERα (**B** and **D**) in human endometrium in the proliferative phase of the menstrual cycle. ERα and COX7RP immunoreactivities were detected in the nucleus and cytoplasm, respectively. Strong immunoreactivities of ERα and COX7RP were detected in the glandular epithelia. Scale bar=100 μm (A, B); 10 μm (C, D).

AF-1, while the E-domain possesses a ligand-dependent AF-2. Biological activities of ERs could be controlled by a number of interacting proteins. The ligand-dependent transactivation of ERs requires the recruitment of coactivators such as TIF2 and SRC-1 (Glass and Rosenfeld, 2000). Transcription activities of ERs are also regulated by phosphorylation. In particular, the serine residue at 118 within the A/B domain of human ERα is a major target site of phosphorylation by MAPK in the presence of growth factors (Kato *et al.*, 1995) and by Cdk7 in a ligand-dependent manner (Chen *et al.*, 2000). Recently, we demonstrated that this serine residue is opposingly dephosphorylated by protein phophatase 5 (Ikeda *et al.*, 2004) (Fig. 2). The protein level of ERα is regulated by the ubiquitin-mediated proteasomal degradation (Nawaz *et al.*, 1999; Tateishi *et al.*, 2004). In addition, some elements in the promoter region have been shown to be responsible for a high expression of ER α in breast cancer cells (Hayashi *et al.*, 1997; Tanimoto *et al.*, 1999). Collectively, it is reasonable to assume that these regulatory mechanisms of ERs are closely associated with oncogenesis and tumor growth. Moreover, it is also indispensable for the diagnosis and treatment of estrogen-associated cancer to reveal the regulatory mechanisms for expression levels of the ER mRNA and protein.

The expression of ERβ has been detected in various tumors including breast cancer (Omoto *et al.*, 2002), uterus

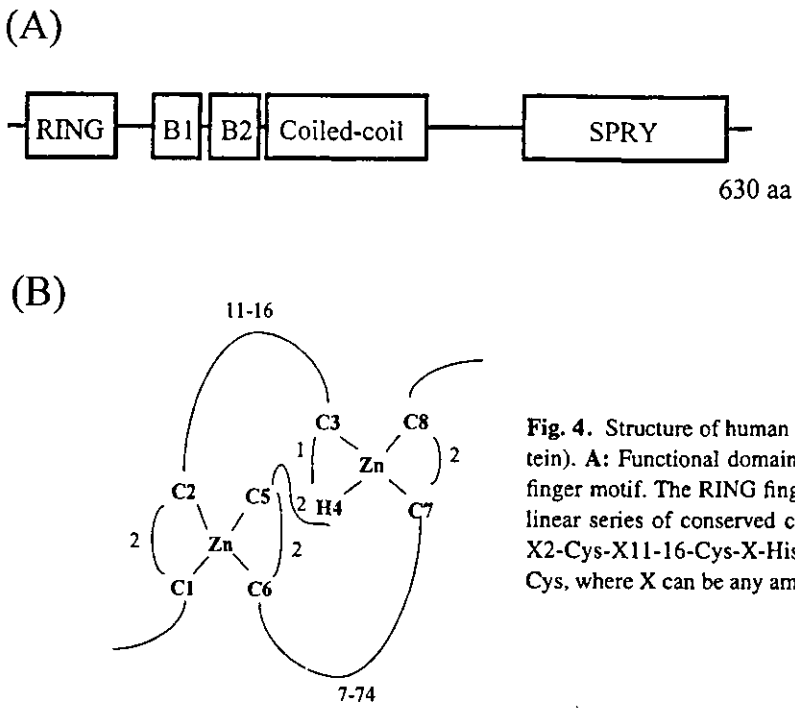

Fig. 4. Structure of human Efp (estrogen-responsive finger protein). **A:** Functional domains of the Efp. **B:** Structure of RING finger motif. The RING finger motif can be defined as a unique linear series of conserved cysteine and histidine residues: Cys-X2-Cys-X11-16-Cys-X-His-X2-Cys -X2-Cys-X7-74-Cys-X2-Cys, where X can be any amino acid.

cancer (Sasano *et al.*, 1999), and prostate cancer (Fujimura *et al.*, 2001). In breast cancer, ERβ shows a tendency to be expressed in ERα-positive carcinomas, while ERα and ERβ double positive cells are also detected. ERβ, as well as ERα, serves as an indicator of a good prognosis in breast cancer (Omoto *et al.*, 2002). It has been found that several variants of ERβ are expressed in breast cancer cells (Leygue *et al.*, 1999). We originally isolated an ERβ isoform, ERβcx (Ogawa *et al.*, 1998), which lacks the last 61 C-terminal amino acids and has an alternative 26 unique amino acids. The ERβcx isoform shows no ligand binding ability and has no capacity to activate transcription in response to estrogen (Ogawa *et al.*, 1998). Moreover, ERβcx shows preferential heterodimerization with ERα rather than with ERβ, inhibiting ERα DNA binding and transactivation. In ERα positive breast cancer, the presence of ERβcx is significantly correlated with the absence of a progesterone receptor (PR) which is a downstream target of activated ER, indicating that ERβcx is a dominant repressor of the ER function in breast cancer. (Saji *et al.*, 2002). These lines of evidence suggest that ERβisoforms are important functional modulators of estrogen-signaling pathways in breast cancer cells and may affect the clinical outcome of patients with breast cancer.

Estrogen-responsive genes in cancer

Estrogen modulates transcription of downstream target genes through ERs. It is thus fundamentally important to identify genes whose expression is regulated by estrogen and to reveal the functions of their protein products. Although a list of ER-target genes has been accumulating, the entire mechanism by which ER enhances the proliferation and progression of tumors remains unknown. In particular, only a few genes are known to be directly regulated by ER through EREs. In order to isolate estrogen-responsive genes having EREs in their transcription regulatory region, we have developed the genomic binding-site cloning (GBSC) method (Inoue *et al.*, 1991, 1999). Using this method, several genomic sequences containing EREs were successfully isolated. Subsequently, novel estrogen-responsive genes were identified nearby the EREs (Inoue *et al.*, 1993; Watanabe *et al.*, 1998). Protein products of these genes include the estrogen-responsive finger protein (Efp), the cytochrome c oxidase subunit VIIa-related polypeptide (COX7RP), and the estrogen receptor-binding fragment-associated antigen 9 (EBAG9).

COX7RP has a well conserved region with cytochrome c oxydase subunit VIIa (Watanabe *et al.*, 1998). Expression of the COX7RP mRNA was up-regulated by estrogen in

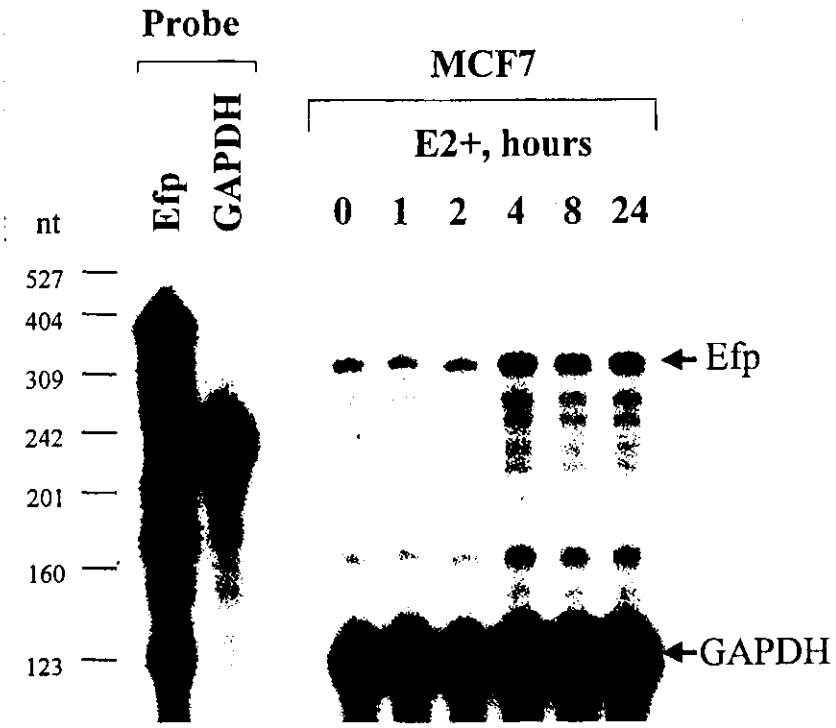

Fig. 5. Estrogen-induced expression of Efp mRNA in MCF7 cells. Total RNAs were isolated from MCF7 cells treated with 10^{-8} M 17β-estradiol at the indicated times. Twenty μg of total RNA was examined by a RNase protection assay using Efp and GAPDH probes. The full-length protected fragment for each probe is indicated.

MCF7 cells. The perfect palindromic ERE found in the first intron possesses an estrogen-dependent enhancer activity in these cells. In addition, an immunohistochemical study demonstrated that the COX7RP protein is co-expressed with the ERα protein in the endometrial glandular epithelium of the human uterus (Fig. 3). We speculate that COX7RP is involved in the regulation of energy production in target cells by estrogen.

Molecular mechanism of Efp function in breast cancer

Among ER-downstream molecules isolated by the GBSC method, we have clarified the molecular mechanism of Efp, which possesses a RING finger motif, two B-boxes, α-helical coiled-coil domains, and a C-terminal SPRY domain (Inoue *et al.*, 1993) (Fig. 4). The RING finger motif is comprised of a unique linear series of conserved cysteine and histidine residues that features a 'cross-brace' arrangement with two zinc ions (Pickart, 2001). Members of the RING finger family grow enormously; some of them have been shown to be responsible for malignant tumors. For instance, PML is responsible for acute premyelocytic leukemia when it forms a fusion protein with the retinoic acid receptor (RAR)α by chromosomal translocation (Jensen *et al.*, 2001). Loss of the tumor suppressor BRCA1 results in chromosomal instability leading to the development of familial breast and ovarian tumors (Ruffner *et al.*, 2001). Efp is predominantly expressed in estrogen target tissues and cells including the mammary gland and uterine epithelial cells (Orimo *et al.*, 1995). Efp is also highly expressed in breast tumors (Ikeda *et al.*, 2000). Expression of the Efp mRNA was shown to be elevated after estrogen treatment in MCF7 cells (Fig. 5). Thus, Efp could function as an estrogen-responsive gene that mediates the estrogen action in cancer. The estrogen-responsive proliferation of the uterine endometrium which expresses abundant ERα was

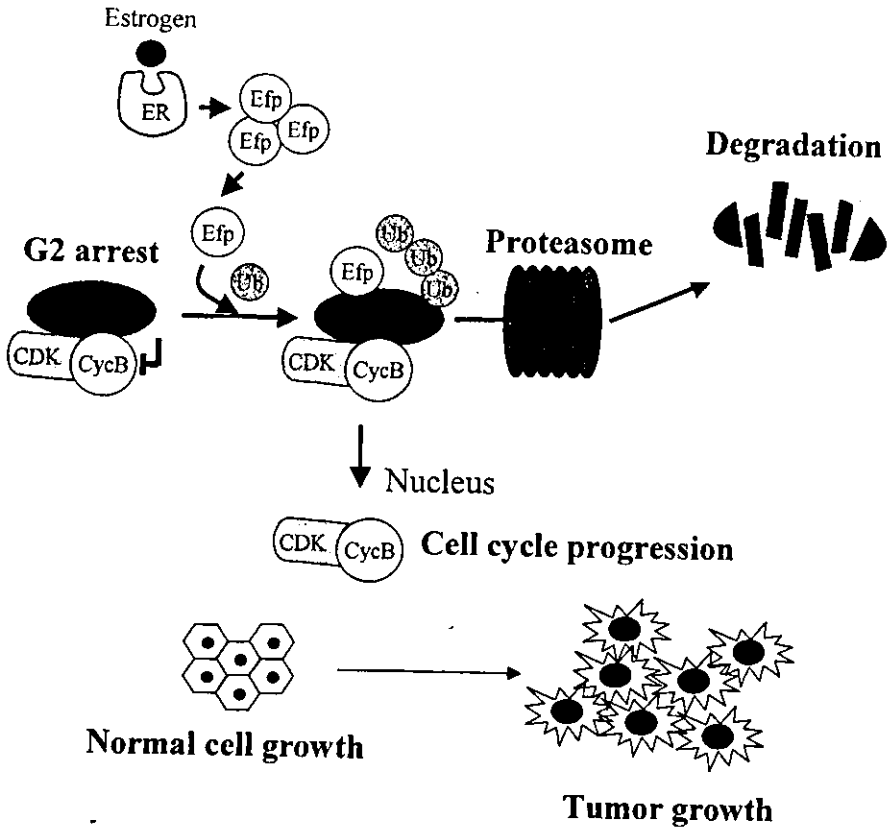

Fig. 6. A simulation model for tumor growth controlled by Efp. The Efp targets 14-3-3σ for proteolysis as a ubiquitin ligase and stimulates tumor growth.

shown to be impaired in Efp knockout mice, suggesting that Efp is a mediator of estrogen-dependent cell growth (Orimo *et al.*, 1999).

To investigate the role of Efp in breast tumor growth, we examined the effects of Efp antisense oligonucleotides on tumor formation in female nude mice inoculated with MCF7 cells (Urano *et al.*, 2002). These mice were ovariectomized or administrated with antisense/sense Efp oligonucleotides. We revealed that the Efp antisense oligonucleotide effectively inhibits the tumor growth generated by MCF7 cells in the recipient mice. MCF7 cells stably expressing Efp (Efp-MCF7) could proliferate even in estrogen-deprived ovariectomized mice. The Efp-MCF7 cells have lower concentrations of the 14-3-3σ protein, which is a negative regulator of the cell cycle progression. The 14-3-3σ protein is important for maintaining G2 arrest by sequestering phosphorylated Cdc2-cyclin B1 from the nucleus into the cytosol (Chan *et al.*, 1999). Interestingly, the expression level of this protein is significantly low in breast tumors (Vercoutter-Edouart *et al.*, 2001; Umbrich *et al.*, 2001). We found that Efp associates with the 14-3-3σ protein. We then demonstrated that Efp functions as a ubiquitin ligase, E3, that ubiquitinates the 14-3-3σ protein, this ubiquitination resulting in the cell cycle progression via the proteasome-dependent degradation of the 14-3-3σ protein (Urano *et al.*, 2002) (Fig. 6).

Perspective

A better understanding of the molecular mechanisms by which estrogen stimulates cell growth can provide new insights into diagnosis, treatment and prevention in estrogen-associated tumors. For this reason, it is indispensable to reveal the expressional and functional regulation of ERs and their target genes. Especially, the identification of estrogen-responsive genes which are closely related to the cancer biology could provide us new approaches for these fields.

Efp, an estrogen-responsive gene, would contribute to the disregulated proliferation of breast cancer cells by the accelerated destruction of a cell cycle regulator, 14-3-3σ. We speculate that Efp could promote tumor growth even in the absence of estrogen and, therefore, the high expression of Efp might be one of reason for acquiring the ability to proliferate independently of estrogen. The future investigation of the relationship between Efp expression and clinical or pathological features could indicate its usefulness as a potential prognostic factor. These trials may lead to the utilization of Efp as a prognostic marker and a therapeutic target in breast cancer. Thus, the accumulation of experimental evidence concerning the estrogen-responsive genes such as Efp can allow us to develop novel cancer treatments separately targeted for each downstream molecule that directly mediates the estrogen action.

Reference

Ali S, Combos RC: Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer* 2: 101-112 (2002).

The ATAC Trialists' Group: Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. *Lancet* 359: 2131-2139 (2002).

Buzdar AU, Hortobagyi GN: Aromatase inhibitors in the treatment of breast cancer, In: *Breast cancer management* (Nabholtz JM, Tonkin K, Aapro MS, Buzdar AU, ed), Martin Dunitz Ltd., London, 2000 (p. 171-180).

Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B: 14-3-3σ is required to prevent mitotic catastrophe after DNA damage. *Nature* 401: 616-620 (1999).

Chen D, Riedl T, Washbrook E, Pace PE, Coombes RC, Egly JM, Ali S: Activation of estrogen receptor α by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. *Mol Cell* 6: 127-137 (2000).

Clarke R, Skaar T, Baumann K, Leonessa F, James M, Lippman J, Thompson EW, Freter C, Brunner N: Hormonal carcinogenesis in breast cancer: cellular and molecular studies of malignant progression. *Breast Cancer Res Treat* 31: 237-248 (1994).

Clemons M, Goss P: Estrogen and the risk of breast cancer. *N Engl J Med* 344: 276-285 (2001).

Early Breast Cancer Trialists' Collaborative Group: Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 351: 1451-1467 (1998).

Fisher ER, Osborne CK, McGuire WL, Redmond C, Knight WA, Fisher B, Bannayan G, Walder A, Jacobson AA, Queen DM, Bennett DE, Ford HC: Correlation of primary breast cancer histopathology and estrogen receptor content. *Breast Cancer Res Treat* 1: 37-41 (1981).

Foster JS, Henley DC, Bukovsky A, Seth P, Wimalasena J: Multifaceted regulation of cell cycle progression by estrogen: Regulation of Cdk inhibitors and Cdc25A independent of cyclin D1-Cdk4 function. *Mol Cell Biol* 21: 794-810 (2001).

Fujimura T, Takahashi S, Urano T, Ogawa S, Ouchi Y, Kitamura T, Muramatsu M, Inoue S: Differential expression of estrogen receptor β(ERβ) and its C-terminal truncated splice variant ERβcx as prognostic predictors in human prostatic cancer. *Biochem Biophys Res Commun* 289: 692-699 (2001).

Glass CK, Rosenfeld MG: The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 14: 121-141 (2000).

Hayashi S-I, Imai K, Suga K, Kurihara T, Higashi Y, Nakachi K: Two promoters in expression of estrogen receptor messenger RNA in human breast cancer. *Carcinogenesis* 18: 459-464 (1997).

Holinka CF, Hata H, Kuramoto H, Gurpide E: Responses to estradiol in a human endometrial adenocarcinoma cell line (Ishikawa). *J Steroid Biochem* 24: 85-89 (1986).

Howell A: New antiestrogens: modulators of estrogen action. In: *Breast cancer management* (Nabholtz JM, Tonkin K, Aapro MS, Buzdar AU, ed), Martin Dunitz Ltd., London, 2000 (p. 143-169).

Ikeda K, Orimo A, Higashi Y, Muramatsu M, Inoue S: Efp as a primary estrogen-responsive gene in human breast cancer. *FEBS Lett* 472: 9-13 (2000).

Ikeda K, Ogawa S, Tsukui T, Horie-Inoue K, Ouchi Y, Kato S, Muramatsu M, Inoue S: Protein phosphatase 5 is a negative regulator of estrogen receptor-mediated transcription. *Mol Endocrinol* 18: 1131-1143 (2004).

Inoue S, Kondo S, Hashimoto M, Kondo T, Muramatsu M; Isolation of estrogen receptor-binding sites in human genomic DNA. *Nucleic Acids Res* 19: 4091-4096 (1991).

Inoue S, Orimo A, Hosoi T, Kondo S, Toyoshima H, Kondo T, Ikegami A, Ouchi Y, Orimo H, Muramatsu M: Genomic binding-site cloning reveals an estrogen-responsive gene that encodes a RING finger protein. *Proc Natl Acad Sci USA* 90: 11117-11121 (1993).

Inoue S, Kondo S, Muramatsu M: Identification of target genes for a transcription factor by genomic binding site (GBS) cloning. In: *Transcription factors - A practical approach*, 2nd ed (Latchman DS, ed), Oxford University Press, New York, 1999 (p.165-178).

Jensen K, Shiels C, Freemont PS: PML protein isoforms and the RBCC/TRIM motif. *Oncogene* 20: 7223-7233 (2001).

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P: Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 270: 1491-1494 (1995).

Leygue E, Dotzlaw H, Watson PH, Murphy LC: Expression of estrogen receptor -1, -2, and -5 messenger RNAs in human breast tissue. *Cancer Res* 59: 1175-1179 (1999).

Millis RR: Correlation of hormone receptors with pathological features in human breast cancer. *Cancer* 46: 2869-2871 (1980).

Nawaz Z, Lonard DM, Dennis AP, Smith CL, O'Malley BW: Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci USA* 96: 1858-1862 (1999).

Nuclear Receptors Committee: A unified nomenclature system for the nuclear receptor subfamily. *Cell* 97: 1-20 (1999).

Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, Ouchi Y, Muramatsu M: Molecular cloning and characterization of human estrogen receptor -cx: a potential inhibitor of estrogen action in human. *Nucleic Acids Res* 26: 3505-3512 (1998).

Omoto Y, Kobayashi S, Inoue S, Ogawa S, Toyama T, Yamashita H, Muramatsu M, Gustafsson J-Å, Iwase H: Evaluation of oestrogen receptor β wild-type and variant protein expression, and relationship with clinicopathological factors in breast cancers. *Eur J Cancer* 38: 380-386 (2002).

Orimo A, Inoue S, Ikeda K, Noji S, Muramatsu M: Molecular cloning, structure, and expression of mouse estrogen-responsive finger protein Efp. Co-localization with estrogen receptor mRNA in target organs, *J Biol Chem* 270: 24406-24413 (1995).

Orimo A, Inoue S, Minowa O, Tominaga N, Tomioka Y, Sato M, Kuno J, Hiroi H, Shimizu Y, Suzuki M, Noda T, Muramatsu M: Underdeveloped uterus and reduced estrogen responsiveness in mice with disruption of the estrogen-responsive finger protein gene, which is a direct target of estrogen receptor α. *Proc Natl Acad Sci USA* 96: 12027-12032 (1999).

Pickart CM: Mechanisms underlying ubiquitination. *Ann Rev Biochem* 70: 503-533 (2001).

Rose PG: Endometrial carcinoma. *N Engl J Med* 335: 640-649 (1996).

Ruffner H, Joazeiro CA, Hemmati D, Hunter T, Verma IM: Cancer-predisposing mutations within the RING domain of BRCA1: loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity. *Proc Natl Acad Sci USA* 98: 5134-5139 (2001).

Saji S, Omoto Y, Shimizu C, Warner M, Hayashi Y, Horiguchi S, Watanabe T, Hayashi S, Gustafsson JA, Toi M: Expression of estrogen receptor (ER) βcx protein in ERα-positive breast cancer: specific correlation with progesterone receptor. *Cancer Res* 62: 4849-4853 (2002).

Sasano H, Suzuki T, Matsuzaki Y, Fukaya T, Endoh M, Nagura H, Kimura M: Messenger ribonucleic acid in situ hybridization analysis of estrogen receptors alpha and beta in human breast carcinoma. *J Clin Endocrinol Meta* 84: 781-785 (1999).

Tanimoto K, Eguchi H, Yoshida T, Hajiro-Nakanishi K, Hayashi S-I: Regulation of estrogen receptor alpha gene mediated by promoter B responsible for its enhanced expression in human breast cancer. *Nucleic Acids Res* 27: 903-909 (1999).

Tateishi Y, Kawabe YI, Chiba T, Murata S, Ichikawa K, Murayama A, Tanaka K, Baba T, Kato S, Yanagisawa J: Ligand-dependent switching of ubiquitin-proteasome pathways for estrogen receptor. *EMBO J* 23: 4813-4823 (2004).

Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S: Hypermethylation of 14-3-3σ (stratifin) is an early event in breast cancer. *Oncogene* 20: 3348-3353 (2001).

Urano T, Saito T, Tsukui T, Fujita M, Hosoi T, Muramatsu M, Ouchi Y, Inoue S: Efp targets 14-3-3σ for proteolysis and promotes breast tumour growth. *Nature* 417: 871-875 (2002).

Vercoutter-Edouart AS, Lemoine J, Le Bourhis X, Louis H, Boilly B, Nurcombe V, Revillion F, Peyrat JP, Hondermarck H: Proteomic analysis reveals that 14-3-3σ is down-regulated in human breast cancer cells. *Cancer Res* 61: 76-80 (2001).

Watanabe T, Inoue S, Hiroi H, Orimo A, Kawashima H, Muramatsu M: Isolation of estrogen-responsive genes with a CpG island library. *Mol Cell Biol* 18: 442-449 (1998).

Wiseman LR, Adkins JC: Anastrozole. A review of its use in the management of postmenopausal women with advanced breast cancer. *Drugs Aging* 13: 321-332 (1998).

Estrogen Receptor-Binding Fragment-Associated Antigen 9 Is a Tumor-Promoting and Prognostic Factor for Renal Cell Carcinoma

Tetsuo Ogushi[1], Satoru Takahashi[1], Takumi Takeuchi[1], Tomohiko Urano[2], Kuniko Horie-Inoue[3], Jinpei Kumagai[1], Tadaichi Kitamura[1], Yasuyoshi Ouchi[2], Masami Muramatsu[3], Satoshi Inoue[2,3]

[1]Department of Urology and [2]Department of Geriatric Medicine, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, JAPAN. [3]Research Center for Genomic Medicine, Saitama Medical School, 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, JAPAN

Financial Support: This work was supported by the Ministry of Health, Labor and Welfare Japan, the Ministry of Education, Culture, Sports, Science and Technology Japan.

Address correspondence to: Satoshi Inoue, Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, JAPAN. Phone: +81-3-5800-8652; Fax: +81-3-5800-6530; E-mail: INOUE-GER@h.u-tokyo.ac.jp

Conflict of interests: none

Running Title: EBAG9 as a Tumor-Promoting Factor for Renal Cell Carcinoma

Key words: EBAG9, renal cell carcinoma, tumor growth, siRNA

Nonstandard abbreviations: estrogen receptor-binding fragment-associated antigen 9 (EBAG9); renal cell carcinoma (RCC)

ABSTRACT

The estrogen receptor-binding fragment-associated antigen 9 (EBAG9) has been identified as a primary estrogen-responsive gene in human breast cancer MCF7 cells. High expression of EBAG9 has been observed in invasive breast cancer and advanced prostate cancer, suggesting a tumor-promoting role of the protein in malignancies. Here we show that intratumoral administration of small interfering RNA (siRNA) against EBAG9 exerted overt regression of tumors following subcutaneous implantation of murine renal cell carcinoma (RCC) Renca cells. Overexpression of EBAG9 did not promote the proliferation of culture Renca cells, however, the inoculated Renca cells harboring EBAG9 (Renca-EBAG9) in BALB/c mice grew faster and developed larger tumors compared with Renca cells expressing vector alone (Renca-vector). After renal subcapsular implantation, Renca-EBAG9 tumors significantly enlarged compared with Renca-vector tumors in BALB/c mice, whereas both Renca-EBAG9 and Renca-vector tumors were developed with similar volumes in BALB/c nude mice. No apparent difference was observed in specific cytotoxic T cell responses against Renca-EBAG9 and Renca-vector cells, nonetheless, the number of infiltrating $CD8^+$ T lymphocytes was decreased in Renca-EBAG9 subcapsular tumors. Furthermore, immunohistochemical study of EBAG9 in 78 human RCC specimens showed that intense and diffuse cytoplasmic immunostaining was observed in 87% of the cases and positive EBAG9 immunoreactivity was closely correlated with poor prognosis of the patients. Multivariate analysis revealed that high EBAG9 expression was an independent prognostic predictor for disease-specific survival ($P = 0.0485$). Our results suggest that EBAG9 is a crucial regulator of tumor progression and a potential prognostic marker for RCC.

INTRODUCTION

Estrogen receptor-binding fragment associated gene 9, EBAG9, is an estrogen-responsive gene that we previously identified in MCF-7 human breast carcinoma cell line using a CpG-genomic binding site cloning method (1). EBAG9 protein, whose molecular size is 32 kDa by Western blot analysis, is expressed in estrogen target organs as well as several other tissues such as brain, liver, and kidney (2). The protein expression of EBAG9 is estrogen-inducible, as it has been shown in ovariectomized mice treated with 17β-estradiol administration (2). The physiological function of EBAG9 has not been well defined, yet the molecule may be implicated in cancer pathophysiology, with several lines of evidence of the protein expression in malignancies including breast (3), ovarian (4), prostate (5), and hepatocellular carcinomas (6). In prostate cancer (5), EBAG9 expression significantly correlated with advanced pathologic stages and high Gleason score ($P = 0.0305$ and $P < 0.0001$, respectively), suggesting the abundance of EBAG9 may relate to the progression of malignant tumors.

In the present study, we investigated whether EBAG9 expression is critical in tumor development of renal cell carcinoma (RCC). RCC that comprises the majority of kidney cancer is one of the ten most common malignancies in industrialized countries (7). The prognosis of patients with advanced RCC is poor, as 5-year survival rate is < 5% (8), and the treatment of metastatic RCC remains a difficult clinical challenge. Development of new and alternative modalities of diagnosis and therapy for RCC is a clinical requisite. We used murine syngeneic renal adenocarcinoma model of Renca cells in this study and investigated whether gene silencing or overexpression of EBAG9 influences Renca cell growth and/or *in vivo* tumorigenesis. Administration of small interfering RNA (siRNA) against EBAG9 regressed subcutaneous Renca tumors. The proliferation of culture Renca cells constitutively expressing EBAG9 was not basically different from control Renca cells, whereas EBAG9-expressing cells grew faster in BALB/c mice and developed larger tumors. The tumor-promoting effect of EBAG9 in Renca tumors may relate to the suppression of antitumor immunity, as intratumoral $CD8^+$ T lymphocytes were reduced in renal subcapsular Renca tumors. The tumorigenic relevance of EBAG9 in Renca models further extended to clinicopathological significance of the molecule in human RCC. EBAG9 immunoreactivity was closely correlated with poor prognosis of the patients and it was an independent prognostic predictor for disease-specific survival. Our findings demonstrate that EBAG9 is a tumor-promoting factor and a potential prognostic marker in RCC.

MATERIALS AND METHODS

Reagents. Rabbit anti-EBAG9 polyclonal antibody was generated against a fusion protein of glutathione-S-transferase and EBAG9 (2). Rabbit polyclonal anti-human CD3 antibody (DAKO Cytomation, Carpinteria, CA), rat anti-mouse CD4 (L3T4)(clone RM 4-5) and rat anti-mouse CD8a (Ly-2)(53-6.7) monoclonal antibodies (BD Pharmingen, San Diego, CA), and anti-β-actin monoclonal antibody (Sigma, St. Louis, MO) were commercially purchased. Human EBAG9 cDNA was cloned into a mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA).

Tumor Cells. Renca is a spontaneously arising murine renal cell carcinoma and was prepared

as previously described (9, 10). Tumor cells were maintained in RPMI1640 containing 10% fetal calf serum and antibiotics.

Mice. BALB/c mice and BALB/c nu/nu mice (Nisseizai, Tokyo, Japan), which were syngeneic to Renca cells, were kept under specific-pathogen-free conditions and fed dry food and water. All mice used for experiments were male at the age of 5 weeks.

Patients and Tissue Preparation. We investigated 78 tissue samples of renal cell carcinoma obtained from patients (14 females and 64 males) who underwent radical or partial nephrectomy at Tokyo University Hospital between the years 1990 and 1995. Patient information was retrieved from the review of patient charts. Staging and grading of the tumors were performed according to the 1997 International Union Against Cancer TNM classification and WHO histopathological typing, respectively (11). The mean age of this population was 54 years old (26-76 years old) and the mean follow-up period was 60 months (2-78 months). For 32 patients with advanced tumors (pT2 or greater), adjuvant therapy was performed including immune therapy (n = 30), radiation (n = 5) and surgery for metastatic diseases in lung, colon, and pancreas (n = 8). During the follow-up period, 55 patients (70.5%) survived without evidence of disease, 8 cases (10.3%) presented with tumor recurrence, and 15 cases (19.2%) died of disease. None died of other diseases.

Western Blot Analysis. Cells were lysed in RIPA buffer [50 mM Tris-HCl, pH8.0, 200 mM NaCl, 20 mM NaF_2, 2 mM EGTA, 1 mM dithiothreitol, 2 mM sodium vanadate, 0.5% v/v Nonidet P-40 supplemented with a protease inhibitor cocktail Complete (Boehringer Manheim GmbH, Mennheim, Germany)] and proteins were resolved by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membranes. Membranes were probed with rabbit anti-EBAG9 antibody or anti-β-actin monoclonal antibody.

Tumor Regression by EBAG9 siRNA. Small interfering RNA (siRNA) duplex that targets EBAG9 was generated by Dharmacon (Lafayette, CO). The target sequence of EBAG9 siRNA was 5'- AAG AAG AUG CAG CCU GGC AAG -3'. Scramble II Duplex (Dharmacon) was used as a non-targeting control siRNA that does not possess homology with known gene targets in mammalian cells. The GC content of Scramble II Duplex was 57.9%, which was identical to that of EBAG9 siRNA.

To investigate *in vivo* silencing effect of EBAG9 siRNA in Renca tumors, intratumoral injection of siRNA duplexes was performed twice every week. Briefly, Renca cells (1×10^4 cells) were implanted in the flank of BALB/c mice. Tumor size measured weekly with a micrometer in two dimensions, and tumor volume was estimated according to the formula: $(\text{smallest diameter})^2 \times (\text{longest diameter})$. When the volumes of tumors reacheds 300 mm^3, siRNA duplexes (10 μg) were injected directly into tumors twice every week, along with 4 μl of GeneSilencer (Gene Therapy System, San Diego, CA) dissolved in 0.1 ml of Opti-MEM (Gibco BRL, Gaithersburg, MD). Mice were sacrified 4 weeks after treatment.

Generation of Renca Cells Stably Expressing EBAG9. Renca cells were transfected with an expression vector pcDNA3 including human EBAG9 cDNA or vector alone using Lipofectamine (Gibco BRL, Gaithersburg, MD). G418-resistent cells were selected and several independent clones were isolated.

Reverse Transcription PCR. Total cellular RNA of Renca cells was extracted using

ISOGEN reagent (NIPPON GENE, Tokyo, Japan) and first-stand cDNA was generated from 5 μg of total cellular RNA using a reverse transcriptase Omniscripit RT™ (QIAGEN, Tokyo, Japan) and random hexamers. To validate the expression of exogenous human EBAG9, reverse transcription (RT) PCR was performed using specific primers for human EBAG9 (sense: 5'-GCTACACAAGATCTGCCTTT-3', antisense: 5'-CTTCTTCATTAGCCGTTGTG-3'). The amplification was performed for 35 cycles at 62°C for annealing, using AmpliGold Taq polymerase (Perkin-Elmer).

***In Vivo* Tumor Challenge.** For subcutaneous implantation, transfected Renca cells (1 x 10^4 cells/ mice) suspended in 0.1 ml of complete medium were injected in the flank of BALB/c mice. Tumor volume was calculated weekly. In survival analyses, Renca-bearing mice were followed up for 14 weeks after implantation.

For renal subcapcular implantation, tumors cells (1 x 10^4 cells/ mice) suspended in 0.1 ml of complete media were inoculated into the subcapsule of the left kidney of BALB/c wild-type and nude mice. Mice were sacrificed 25 days after implantation and tumors were exicised.

Cell Proliferation Assay. Cells were seeded at a density of 1-3 x 10^5 cells/dish into 10-cm dishes and hemocytometer counting was performed every two days. Doubling time during exponential growth was determined by a formula: [incubation time (h) x $\log_{10}2$]/ [$\log_{10}$(cell number at sampling period) - $\log_{10}$(plating cell number)](12).

Proliferation assays were performed using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8) reagent (Nacalai, Kyoto, Japan)(13). The assay is based on the conversion of the MTT-like tetrazolium salt WST-8 to a water-soluble formazan by metabolically active cells, provides a quantitative determination of viable cells. Cells were seeded in 96-well plates at an initial density of 625-5,000 cells/well. At one hour after inoculation, cells were transfected with either EBAG9 siRNA or Scramble II Duplex (100 ng/well) using GeneSilencer reagent (Gene Therapy Systems). Assays were performed on days 0, 2, and 4. For cells cultured up to day 4, medium was once exchanged on day 2. Spectrophotometric absorbance at 450 nm (for formazan dye) was measured with absorbance at 620 nm for reference.

Cytotoxicity Assay. Renca-EBAG9 or Renca-vector cells were used as target cells. Splenocytes of Renca-bearing BALB/c mice were stimulated for 5 days *in vitro* with irradiated Renca cells at a splenocyte: tumor cell ratio of 20:1 in the presence of 1,000 IU/ml interleukin-2, and used as effector CTLs. Target cells were incubated with effector CTLs at various E/T ratios in a final volume of 200 μl for 18 h at 37°C. Lactate dehydrogenase release from cells with a damaged membrane was examined using CytoTox-ONE Reagent (Promega, Madison, WI) and fluorescence was measured with an excitation wave length of 560 nm and an emission wave length of 590 nm. Experiments were performed in triplicate.

Immunohistochemistry. Immunohistochemical studies were performed using the streptavidin-biotin amplification method with horseradish peroxidase detection. Paraffin sections of tumors were blocked in 0.3% H_2O_2 (30 min) and in 10% FCS (30 min), incubated overnight with specific antibodies against CD3, CD4, or CD8a for Renca tumors (1:20 dilution), or with purified rabbit anti-EBAG9 antibody for human RCC (1:40 dilution). Sections were incubated with biotinylated rabbit ani-rat IgG or anti-rabbit EnVison$^+$ reagent (Dako), developed by

diaminobenzidine (Sigma) and counterstained with hematoxylin (Sigma). Negative controls were performed for each slide, using nonimmune IgG.

In Renca experiments, numbers of tumor infiltrating-lymphocytes (TILs) positive for CD3, CD4, or CD8 expression were microscopically examined in the high power field (HPF) of view at a magnification of 400X (14). BALB/c mouse spleen specimen was used as a positive control.

In RCC examination, immunoreactivity (IR) scores of EBAG9 expression were determined by two pathologists according to percentages of positive cells. Human breast cancer section (DAKO) was used as a positive control. Positivity was 0-4% for IR score 0 (negative), 5-24% for 1+, 25-49% for 2+, 50-100% for 3+. Sections that had ≥ 25% positive cells but apparent lower intensity compared with positive controls were scored as IR score 1+. IR scores 1+, 2+, and 3+ were defined as positive staining. If IR scores were different between two pathologists, the average IR score was adopted. If several types of histology were included in one section, IR score of predominant histology was utilized.

Statistical Analyses. Comparisons between different groups of Renca samples were analyzed with non-parametrical Mann-Whitney U test. The associations between EBAG9 immunoreactivity and clinicopathological characteristics were evaluated by student-*t* test or Fisher's exact probability test. Disease-specific survival was computed by Kaplan-Meier method and the curves were compared by log-rank test. Multivariate analysis of prognostic factors was performed using Cox-proportional hazard regression model. Computations were done with the StatView 5.0J software (SAS Institute Inc., Cary, NC). All *P* values are two-sided and evaluated as significant if < 0.05.

RESULTS

Gene silencing of EBAG9 suppressed *in vivo* tumor growth of Renca cells. To determine the role of EBAG9 in tumor growth of renal cancer cells, we investigated the effects of synthesized siRNA duplexes targeting EBAG9 on subcutaneous tumor models of Renca cells implanted in syngeneic BALB/c mice. Intratumoral injection of EBAG9 siRNA reduced the protein levels of endogenous EBAG9 compared with the levels of EBAG9 in parental Renca cells or in the Renca tumor treated with control scrambled siRNA duplexes (Fig. 1A). Under the treatment of scrambled siRNA, subcutaneously implanted Renca cells developed prominent tumors, whereas the injection of EBAG9 siRNA suppressed tumor growth of Renca cells (Fig. 1B and 1C). After 4-week treatments, the volume of tumors with EBAG9 siRNA treatment was significantly smaller than that with scrambled siRNA (3,854 ± 665 mm^3 *versus* 6,315 ± 1,053 mm^3, n = 5; $P = 0.0472$). We infer that tumor growth is modulated by EBAG9 expression, implicating EBAG9 as a tumor-promoting factor in renal carcinoma.

Generation of Renca cells stably expressing EBAG9. To explore whether constitutive EBAG9 expression influences tumor growth, we generated Renca cells stably expressing human EBAG9. We selected two Renca-EBAG9 cell clones #3 and #4 that express human EBAG9 mRNA as comfirmed by RT-PCR using human EBAG9 specific primers (Fig. 2A, top panel). The amounts of EBAG9 proteins in Renca-EBAG9 cells were ~2.0 fold increased compared with those in

parental Renca cells and Renca-vector cell clones #1 and #2, that were transfected with pcDNA3 empty vector (Fig. 2A, bottom panel). In terms of cell growth rate, doubling time of culture Renca-EBAG9 cells was not significantly different from that of Renca-vector cells (Fig. 2B). Proliferation of Renca cells was further analyzed by a colorimetric MTT-like assay using a tetrazolium monosodium salt WST-8 that is converted to a water-soluble formazan by metabolically active cells (Fig. 2C). Neither EBAG9 overexpression nor RNA interference against EBAG9 did not significantly influence the growth of Renca cells. Moreover, EBAG9 overexpression did not influence the incorporation of BrdU in culture Renca cells (data not shown). The results suggest that stable expression of EBAG9 itself does not accelerate the proliferation of culture tumor cells.

EBAG9 promotes *in vivo* tumor growth of Renca cells. In spite of little difference of propagation abilities between Renca-EBAG9 cells and Renca-vector cells in culture, Renca-EBAG9 cells subcutaneously implanted into BALB/c mice developed more than 4-fold larger tumors compared with Renca-vector cells at 4 weeks after inoculation (Fig. 3A and 3B). Mean tumor volumes at 4 weeks were 1,712 ± 506 mm^3 for Renca-EBAG9 cell clones #3 and #4 *versus* 366 ±110 mm^3 for Renca-vector cell clones #1 and #2 ($P = 0.0055$, Fig. 3B).

In terms of prognosis of mice harboring Renca tumors, 23.5% of mice with Renca-vector cells (n = 17) survived on day 100 after tumor challenge whereas only 5.6% of mice with Renca-EBAG9 cells (n = 18) survived at the same period (Fig, 3C, $P = 0.0412$ by log-rank test). Systemic metastases, including tumor dissemination into peritoneum and distant metastases of lung and liver, were reasons for death in all deceased cases.

EBAG9 suppresses host immune surveillance. To determine whether aberrant EBAG9 expression in Renca cells affects the local immune responses in tumors, we implanted Renca-EBAG9 cells or Renca-vector cells under the renal capsule of BALB/c mice and immunodeficient BALB/c nude mice. Both Renca cell lines formed macroscopic tumors in all of the cancer-bearing hosts by Day 25 (Fig. 4A). In conventional BALB/c mice, Renca-EBAG9 tumors grew significantly larger compared with Renca-vector tumors (Fig. 4A and 4B). Mean volumes of tumors on Day 25 in BALB/c mice were 856 ± 162 mm^3 (n = 19) for Renca-EBAG9 clones #3 and #4 *versus* 149 ± 59 mm^3 (n = 18) for Renca-vector clones #1 and #2 (Fig. 4B, $P < 0.0001$). In immunodeficient BALB/c nude mice, both Renca-vector cells and Renca-EBAG9 cells developed extensive tumors compared with tumors in BALB/c mice and there was no significant difference in tumor volumes between Renca-vector cells and Renca-EBAG9 cells (Fig. 4A and 4B). Mean volumes of tumors on Day 25 in BALB/c nude mice were 2,215 ± 227 mm^3 (n = 18) for Renca-EBAG9 clones #3 and #4 versus 1,802 ± 240 mm^3 (n = 23) for Renca-vector clones #1 and #2 (Fig 4B, $P = 0.118$). These results may suggest that aberrant EBAG9 expression in Renca cells hampers a local primary immune response that retards the growth of tumors, rather than potentiates the intrinsic tumorigenicity of the tumor cells.

To investigate whether the progression of Renca-EBAG9 tumors depends on a reduced sensitivity of the cells to tumor-specific CTLs, we performed cytotoxicity assays. Effector CTLs were derived from splenocytes of Renca-bearing BALB/c mice, after 5-day restimulation with Renca cells in the presence of interleukin-2. (Fig. 4C). Renca-EBAG9 cells and Renca-vector cells were equally lysed by tumor-specific CTLs, suggesting that EBAG9 expression itself does not

affect the sensitivity of Renca cells to CTL lysis.

To assess whether EBAG9 modulates the subtype-specific reactivity of T lymphocytes against tumors, we examined the numbers of TILs in renal subcapsular Renca tumors developed in BALB/c mice (Fig. 4D). No significant differences in numbers of $CD3^+$ and $CD4^+$ T cells were observed between Renca-vector and Renca-EBAG9 tumors, whereas the number of $CD8^+$ T cells in Renca-EBAG9 tumors was significantly decreased compared with that in Renca-vector tumors ($P < 0.05$).

Expression of EBAG9 protein in human RCC tumors. The finding that EBAG9 modulated the growth of Renca tumors led us to the notion whether the molecule contributes to the progression of renal cell carcinoma in human tissues. EBAG9 expression was evaluated immunohistochemically in 78 RCC whole tissue specimens including normal lesions. In non-carcinomatous lesions, a weak and scattered immunostaining of EBAG9 was observed in the cytoplasm of the mesangial cells (Fig. 5A) as well as on the luminal surface of the renal tubular cells (data not shown). The levels of EBAG9 expression in normal renal tissues corresponded to IR score 0. In RCC tumors, 10 of 78 cases (13%) had negative immunoreactivity of EBAG9, whereas 68 of cases (87%) showed EBAG9 positivity. In regard to EBAG9-positive RCC tumors, the cancer cells generally retain intense and diffuse staining patterns in the cytoplasm or on the membrane (Fig. 5B, C, and D). The levels of EBAG9-positivity were IR score 1+ for 18 RCC tumors (23%), 2+ for 31 tumors (40%), and 3+ for 19 tumors (24%). With respect to RCC histology, clear cell tumors displayed an intense membrane staining as well as a diffuse cytoplasmic staining of EBAG9 (Fig 5B, IR score 2+). Sarcomatoid tumors showed an intense and frequent cytoplasmic immunoreactivity (Fig 5C, IR scores 3+). Lung metastatic tumors showed the highest EBAG9 staining, predominantly in the cytoplasm (Fig 5D, IR score 3+).

A significant association between EBAG9 immunoreactivity and clinicopathological parameters was observed in RCC patients (Supplementary Table 1). EBAG9 positivity (IR score >1+) was significantly correlated with advanced pathological tumor stages, positivity of vascular infiltration, and non-clear cell histology ($P = 0.0017$, $P = 0.0109$, and $P = 0.0126$, respectively).

In Kaplan-Meier analysis of the RCC patients, those in which the tumor had high EBAG9 immunoreactivity (IR score 3+) showed a shorter disease-specific survival (Fig. 6) compared with patients showing low or negative EBAG9 immunoreactivity (IR score 0–2+). The 5-year disease-specific survival in cases with EBAG9 IR score 3+ was 55% whereas 91.2% of patients with low or negative EBAG9 immunoreactivity were alive during the same period.

In univariate Cox proportional hazards analysis for 5-year disease-specific survival, established prognostic factors including infiltration, pathological stage, and metastatic status are the most significant univariate parameters of survival (Supplementary Table 2; $P < 0.0001$ for all). Lower EBAG9 immunoreactivity as well as negativity of lymph node status or vascular infiltraion are also involved in significant univariate survival predictors ($P = 0.0007$, 0.0002, and 0.0003, respectively). In multivariate Cox proportional hazards analysis, negative metastatic status is the most significant predictor of survival (Supplementary Table 3; $P < 0.001$; relative risk, 42.53) Notably, high EBAG9 immunoreactivity is associated with disease-specific death in multivariate analysis ($P = 0.0485$; relative risk, 5.09). These results indicate that high immunoreactivity of EBAG9 is a potential poor prognostic parameter in RCC patients.