Transcription factor EGR3 is involved in the estrogen-signaling pathway in breast cancer cells

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Abstract

Estrogen has been closely associated with the genesis and malignant progression of breast cancer. However, the molecular mechanism underlying the effects of estrogen is far from being completely clarified. We previously developed a custom-made cDNA microarray consisting of approximately 200 estrogen-responsive genes in breast cancer cells. Using this system, we found one estrogen-induced gene in various cancer cell lines, including breast cancer MCF-7 cells, which encode a zinc-finger transcription factor, EGR3 (early growth response 3). Northern blot analysis of estradiol-treated MCF-7 cells showed rapid and robust induction of *Egr3*, and addition of cycloheximide or ICI 182,780 suggested that *Egr3* is the *bona fide* target for the estrogen receptor α (ERα). Using stable transformants derived from MCF-7 cells which were transfected with expression-controllable *Egr3*-expression vector, we demonstrated that *Nab2* is one of the target genes for EGR3. Microarray analysis of the transformants revealed other candidate EGR3-induced genes. These strategies could be useful for analyzing downstream genes of ERα, and may contribute to elucidating the extensive signaling network of estrogen stimuli. Furthermore, a reporter assay using the upstream region of *fasL* probably involving escape from the immune system revealed that *fasL* is another target gene for EGR3. The roles of EGR3 in the physiology of breast cancer are discussed.

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Introduction

Estrogen plays an important role in the development of mammary glands and the genesis, growth and progression of breast cancer (Pike *et al.* 1993, Fanelli *et al.* 1996, Anderson *et al.* 1998). In normal and malignant mammary cells, estrogen receptor alpha (ERα), one of the nuclear receptor transcription factors, is the primary target of estrogen, and the enhanced expression of ERα is one of the most frequent events in the development of breast cancer (Hayashi *et al.* 1997, Shoker *et al.* 1999).

There are many reports concerning the target genes transcriptionally activated by ERα, such as *pS2* (Masiakowski *et al.* 1982), cathepsin D (Westley & Rochefort 1980) and *Efp* (Ikeda *et al.* 2000). Recently, gene-expression profiling of mammary tumors and breast cancer cells by microarray analysis has made an enormous contribution to expanding the list of novel estrogen-responsive genes (Finlin *et al.* 2001, Soulez & Parker 2001, Bouras *et al.* 2002). At least some of those genes have been investigated regarding their function in breast cancer, and their significance in relation to the physiology of mammary tumors has been discussed (Finlin *et al.* 2001, Prest *et al.* 2002, Urano *et al.* 2002). Nonetheless, the entire mechanism of the pathway from ERα leading to the physiological effects on normal and malignant mammary cells is far from being completely clarified.

In a previous report, we developed a custom-made cDNA microarray system consisting of approximately 200 estrogen-responsive genes (Inoue *et al.* 2002). Gene-expression profiling of human cancer cells with this microarray revealed that a gene encoding the zinc-finger transcription

factor EGR3 (early growth response 3) was induced by estrogen in breast cancer MCF-7 cells and other cell lines derived from several other organs. Our novel findings concerning the estrogen-dependent expression of *Egr3* suggest that EGR3 may play a key role in the estrogen-signaling pathway in a variety of organs, expanding the intracellular influence of estrogen through the transactivation of downstream target genes.

EGR3 belongs to the EGR family and shares a common sequence called the EGR response element with other members involved in DNA-binding and transactivation (O'Donovan *et al.* 1999). The activities of the members except for EGR4 are regulated by the specific cofactors NAB1 (Russo *et al.* 1995) and NAB2 (Svaren *et al.* 1996). EGR1, a prototype of the family, has been reported to be induced by diverse stimuli, including the addition of mitogens (Gashler & Sukhatme 1995), and is considered to be involved in either cell proliferation or differentiation in a variety of tissues and cell lines. For example, EGR1 was reported to be essential for the transcriptional autoregulation of basic fibroblast growth factor (bFGF) in hepatoma cells (Wang *et al.* 1997), and in the case of PC12 cells, EGR1 was considered to be involved in differentiation induced by nerve growth factor (NGF), and the overexpression of cofactor NAB2 inhibited differentiation of the cells (Qu *et al.* 1998). EGR3 was also reported to be induced by various stimuli, a large number of which were the same as the case of EGR1 (O'Donovan *et al.* 1998, Mercier *et al.* 2001, Jouvert *et al.* 2002). Moreover, EGR3 has been shown to be essential for the development of muscle spindle in mice (Tourtellotte *et al.* 2001), and knockout mice (*Egr3* −/−) exhibited several symptoms, such as gait ataxia and scoliosis, presumably due to the lack of muscle spindle (Tourtellotte & Milbrandt 1998). On the other hand, *fasL* has previously been reported to be induced under the control of EGR3 in T cells activated in a TCR-mediated manner or by phorbol ester plus a Ca^{2+} ionophore (Mittelstadt & Ashwell 1998); *fasL* is the only target gene for EGR3 so far. In contrast, there has been no report regarding the expression and function of *Egr3* in mammary gland and breast cancer. However, these findings regarding EGR3 led us to hypothesize that EGR3 may have an important role in the physiology of normal and malignant mammary cells, through being induced by estrogen

and in turn inducing the expression of other estrogen-responsive genes, some of which were identified by our microarray analysis (Inoue *et al.* 2002).

This report addresses the role of *Egr3* as an intracellular mediator of the estrogen-signaling pathway in breast cancer.

Materials and methods

Cell culture

Human breast cancer MCF-7 cells were maintained in RPMI 1640 medium (NISSUI Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Tissue Culture Biologicals, Tulare, CA, USA). For treatment of the cells with estrogen, the medium was replaced with phenol red-free RPMI 1640 medium (Sigma, St Louis, MO, USA) containing FBS treated with dextran-coated charcoal (DCC-FBS). All the culture media contained 2 mM glutamine (Sigma) and 40 µg/ml gentamicin (Schering-Plough, Osaka, Japan). All cells were incubated at 37 °C in humidified air containing 5% CO_2. MCF-7 Tet-Off cells (Clontech) and stable transformants derived from them were cultured as in the case of parental MCF-7 cells, except that 100 µg/ml of geneticin and 50 µg/ml of hygromycin (only for stable transformants) were added in each medium.

Northern blot analysis

For Northern blot analysis, MCF-7 cells were grown in estrogen-starved medium for 5–7 days and treated with ethanol (E_2^-) or 10 nM 17β-estradiol (E_2^+) for the periods specified below. To examine the effects of estrogen antagonists, 1 µM of 4-hydroxytamoxifen (Sigma) or ICI 182,780 (AstraZeneca Pharmaceuticals, Stanhope Gate, London, UK) was added to each medium together with 10 nM of 17β-estradiol. Cycloheximide was added at the concentration of 10 µg/ml concurrently with ethanol or 17β-estradiol. Total RNA was prepared from each cell culture by the method of Chomczynski and Sacchi (1987). For each sample, 20 µg total RNA were used for Northern analysis. DNA fragments (420–520 bp) for hybridization probes were prepared by RT-PCR, using total RNA from MCF-7 cells as a template and the following primers: 5′-CCA GGA TTA CCA ATC

GGC C-3′ and 5′-GCA GGC AAA GGG CTT CTC G-3′ for *Egr3* (Mages *et al.* 1993); 5′-AGC TCA CCC GCC ACA TCC-3′ and 5′-CGC TGA AGG AGT TGG TGA C-3′ for *Egr1* (Suggs *et al.* 1990); and 5′-ATC AAC GAG GCT GCT GCC-3′ and 5′-CCT TCC TCG AAC TCT GCG-3′ for *Nab2* (Kirsch *et al.* 1996). The primers for *pS2* and β-actin have been described previously (Inoue *et al.* 2002). RT-PCR, labeling of the probes and hybridization were performed as described previously (Inoue *et al.* 2002).

Isolation of stable transformants with inducible *Egr3* expression

A 1·2 kb cDNA fragment containing the full-length coding region for *Egr3* (Mages *et al.* 1993) was amplified by RT-PCR, using total RNA from MCF-7 cells as described above and the following primers: 5′-GCG GGATCC GAG TGC TAT GAC CGG CAA AC-3′ and 5′-CCG GGATCC CCG ATC CTC AGG CGC AGG-3′ (BamHI sites underlined). The *Egr3* cDNA fragment was digested with BamHI and subcloned into the expression vector pTRE2hyg (Clontech) with the same orientation as that of the promoter. The resultant plasmid named pTRE2hyg:EGR3 and the empty vector pTRE2hyg were transfected into MCF-7 Tet-Off cells (Clontech), using *Trans* IT Polyamine Transfection Reagents (Takara, Tokyo, Japan) according to the manufacturer's instructions with some modifications. Briefly, MCF-7 Tet-Off cells were grown in the indicated media to 30–60% confluence. Each plasmid DNA was mixed with Trans IT LT-1 reagent in serum-free medium and added to the culture. After 2-day incubation, 100 μg/ml of hygromycin were added to the culture and incubated for about 2 weeks to select stable transformants. Single colonies were picked up and grown in the indicated media containing 100 μg/ml of geneticin and 50 μg/ml of hygromycin.

Analysis of gene expression in stable transformants

MCF-7 Tet-Off cells stably transfected with pTRE2hyg or pTRE2hyg:EGR3 were grown in the indicated media, to which 50 ng/ml of doxycycline were added. Afterwards, the media were changed and the culture was continued for 48 h in the presence or absence of 50 ng/ml of

doxycycline. Isolation of total RNA and Northern blotting for several genes were performed as described above. For gene-expression profiling by EstrArray (InfoGenes Co., Tsukuba, Ibaraki, Japan), which is the improved version of the custom-made microarray formerly named InfoArray (Inoue *et al.* 2002) (InfoGenes), stable transformants were grown in estrogen-starved medium for 2 days. The medium was then replaced, and the cells were treated with 100 ng/ml of doxycycline or H_2O (control) for 72 h. Poly $(A)^+$ RNA was isolated from the cells untreated and treated with doxycycline, and was used for preparation of Cy3- and Cy5-labeled cDNA probes respectively. Labeling and hybridization of the cDNA probes into EstrArray were done according to the procedure described previously (Inoue *et al.* 2002) and the manufacturer's instructions. For comparison, the estrogen-responsive gene-expression pattern of parental MCF-7 cells was also analyzed by EstrArray, following the reported procedure (Inoue *et al.* 2002) and the manufacturer's instructions. Scanning and data analysis were performed as described previously (Inoue *et al.* 2002), except for the following modification. In the case of the stable transformants, the data for the spots with poor hybridization (signal areas of either Cy3 or Cy5 below 100) were removed from the data processing, to improve the correlation coefficients between duplicated sets of $\log_2(Cy3/Cy5)$ values. In the case of parental MCF-7 cells, there was actually no need to cut off the data for improvement of the correlation coefficients (0·968 without filtration of the data).

Luciferase reporter assay

A 0·5 kb DNA fragment (-347 to $+175$ in base pairs from the transcription initiation site) containing the upstream region of *fasL* (Holtz-Heppelmann *et al.* 1998) was amplified with LA *Taq* polymerase (Takara), using the following primers: 5′-GCG GCTAGC GTT GTG TAT ATG GTA GAA TTC-3′ (NheI site underlined) and 5′-CCG AAGCTT CTG GTG AGT CAG GCC AGC-3′ (HindIII site underlined). The amplified DNA fragment was digested with NheI and HindIII and subcloned into a reporter vector pGL3-Basic (Promega, Madison, WI, USA). The resultant plasmid was named pGL3-FasL 0·5 kb. For construction of the *Egr3*-expression plasmid, the expression vector pRc/CMV (Invitrogen, Tokyo,
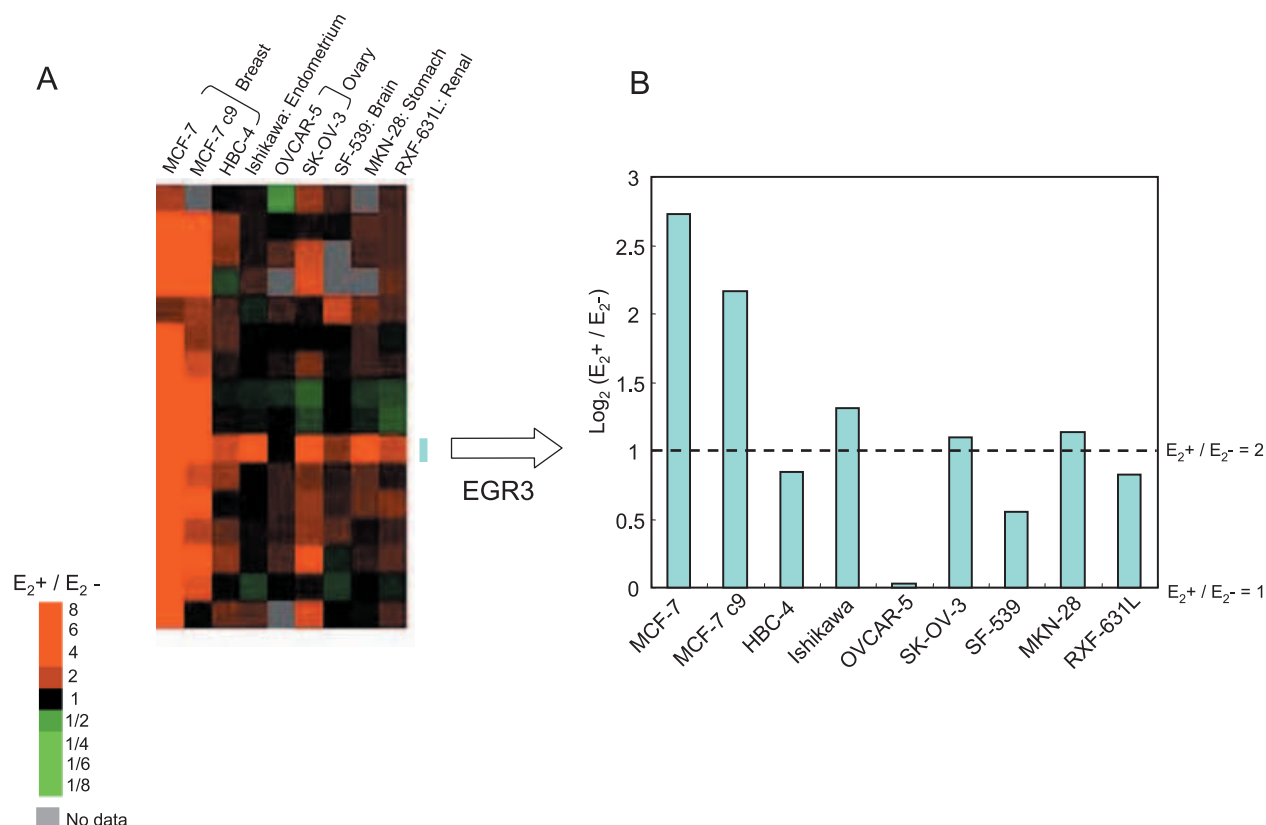
Figure 1 (A) Estrogen-responsive expression patterns of a gene cluster including *Egr3* in breast cancer MCF-7 cells and other cell lines derived from a variety of organs. Patterns are represented as a pseudocolor visualization matrix, an extract from Fig. 6 in the previous report (Inoue *et al.* 2002). The color scale used to represent the expression ratios is shown on the lower left. The gray color means that there are no reliable data after filtrating due to poor hybridization. The expression pattern of *Egr3* is indicated in (B). (B) Expression ratios (E_2^+/E_2^-) of *Egr3* in all cell lines examined are shown in a logarithmic scale.

Japan) was digested with HindIII, polished with the Klenow Fragment, and 5′-dephosphorylated with alkaline phosphatase. The BamHI-digested 1·2-kb cDNA fragment encoding *Egr3* described above was also polished with the Klenow Fragment and subcloned into pRc/CMV with the same orientation as that of the promoter. The resultant plasmid was named pRc/CMV:EGR3. These plasmids were transiently transfected into MCF-7 cells cultured under estrogen-starved conditions for 2 days, using *Trans* IT Polyamine Transfection Reagents (Takara) as described above, together with an internal control vector pRL-TK (Promega). After incubation for 30 h, the luciferase activity of each cell lysate was measured with the Dual-Luciferase Reporter Assay System (Promega), and each value was normalized to the internal *Renilla* luciferase activity as a control.

Results

E$_2$-responsive expression of *Egr3* and related genes in MCF-7 cells

Previously, we developed a custom-made cDNA microarray system consisting of approximately 200 estrogen-responsive genes (Inoue *et al.* 2002). Estrogen-responsive gene-expression profiling of human cancer cells using the microarray revealed that a gene encoding a zinc-finger transcription factor EGR3 was induced by 17β-estradiol treatment in breast cancer MCF-7 cells and several other cell lines derived from a variety of organs (Fig. 1), implying the significance of *Egr3* as one of the key molecules in the estrogen-signaling pathway.

At first, we performed Northern blot analysis of *Egr3* expression in breast cancer MCF-7 cells to

confirm the results from microarray analyses and to investigate the time course of estrogen-responsive expression of the gene. As shown in Fig. 2, expression of *Egr3* mRNA was induced 6 h after estrogen treatment, reaching a maximal induction level (10–20-fold) at 24–72 h after the treatment. This observation is similar to the expression patterns of the early-responsive estrogen-induced genes, such as *pS2* and insulin-like growth factor-binding protein 4 (IGFBP4), described in our previous report (Inoue *et al.* 2002).

The estrogen-responsive expression pattern of several genes related to *Egr3* was also examined (Fig. 2). *Egr1*, the prototype of the EGR family, exhibited small fluctuations in the expression levels at each time point, but is not likely to be regulated by estrogen. *Nab2,* one of the cofactors specific for EGR family members, was induced by estrogen, as in the case of *Egr3*, but the start of substantial induction was apparently later than that of *Egr3*. On the other hand, another cofactor for EGR family members, *Nab1,* did not show estrogen-responsiveness (data not shown).

Effects of antiestrogens and cycloheximide on E$_2$-responsive expression

For further investigation, the effects of estrogen antagonists were analyzed. As shown in Fig. 3A, both the estrogen antagonists 4-hydroxytamoxifen (OHT) and ICI 182,780 severely inhibited *Egr3* induction by estrogen. The extent of inhibition was much higher by ICI 182,780 than OHT. Similar observations were also obtained concerning the expression pattern of *Nab2*. The effects of cycloheximide, a protein synthesis inhibitor, were also examined, and the results are shown in Fig. 3B. *Egr3* exhibited a significant level of induction in response to estrogen even in the presence of cycloheximide, suggesting that the expression of *Egr3* mRNA does not require *de novo* protein synthesis. These observations regarding *Egr3* are similar to the case of *pS2*, which has been demonstrated to be one of the primary targets for estrogen receptor α (ERα) (Brown *et al.* 1984, Stack *et al.* 1988). In contrast, *Nab2* was not induced by estrogen in the presence of cycloheximide, suggesting that another *de novo* synthesized protein is at least in part essential for the expression of *Nab2*.

## Identification of *Nab2* as a downstream target gene for EGR3

To investigate the function of *Egr3*, full-length *Egr3* cDNA was cloned into the expression vector pTRE2hyg (Clontech) and stably transfected into MCF-7 Tet-Off cells (Clontech). The combination of pTRE2hyg with MCF-7 Tet-Off cells makes it possible to regulate transcriptionally a transfected gene by tetracycline or its analog doxycycline. Several isolated clones transfected with *Egr3*-expression vector and one control clone transfected with empty vector were cultured in the presence or absence of doxycycline for 2 days, and Northern blot analysis of *Egr3* expression was performed (Fig. 4). As expected, all the clones transfected with *Egr3*-expression vector demonstrated overexpression of *Egr3* mRNA in the absence of doxycycline, and its addition drastically repressed *Egr3* expression, whereas a control clone with empty vector showed no regulation of *Egr3* expression. Moreover, Northern blot analysis revealed that the levels of *Nab2* mRNA were also under the control of doxycycline in *Egr3*-expressing transformants (Fig. 4). In all the *Egr3*-expressing clones examined, *Nab2* was upregulated in the absence of doxycycline, that is, under the conditions allowing overexpression of *Egr3*. This suggests that *Nab2* is one of the downstream target genes for EGR3.

Microarray analysis using *Egr3*-expressing transformants

To search for other target genes of EGR3 and investigate its contribution as a signal mediator in the estrogen signal network, we performed gene-expression profiling of *Egr3*-expressing transformants. For this purpose, we developed a microarray named EstrArray (InfoGenes), an updated version of the custom-made microarray previously reported (Inoue *et al.* 2002), replacing 28 genes in the former version with the same number of other clones which have been identified as estrogen-responsive genes through GeneChip analysis (Affymetrix) of MCF-7 cells (data not shown). Poly (A)$^+$ RNA was isolated from the transformants cultured in the presence or absence of doxycycline, and the gene-expression patterns were analyzed with EstrArray (Fig. 5). Comparison of the gene-expression profiles of *Egr3*-expressing clones with estrogen-responsive gene-expression patterns in parental MCF-7 cells revealed candidate target genes of EGR3, which
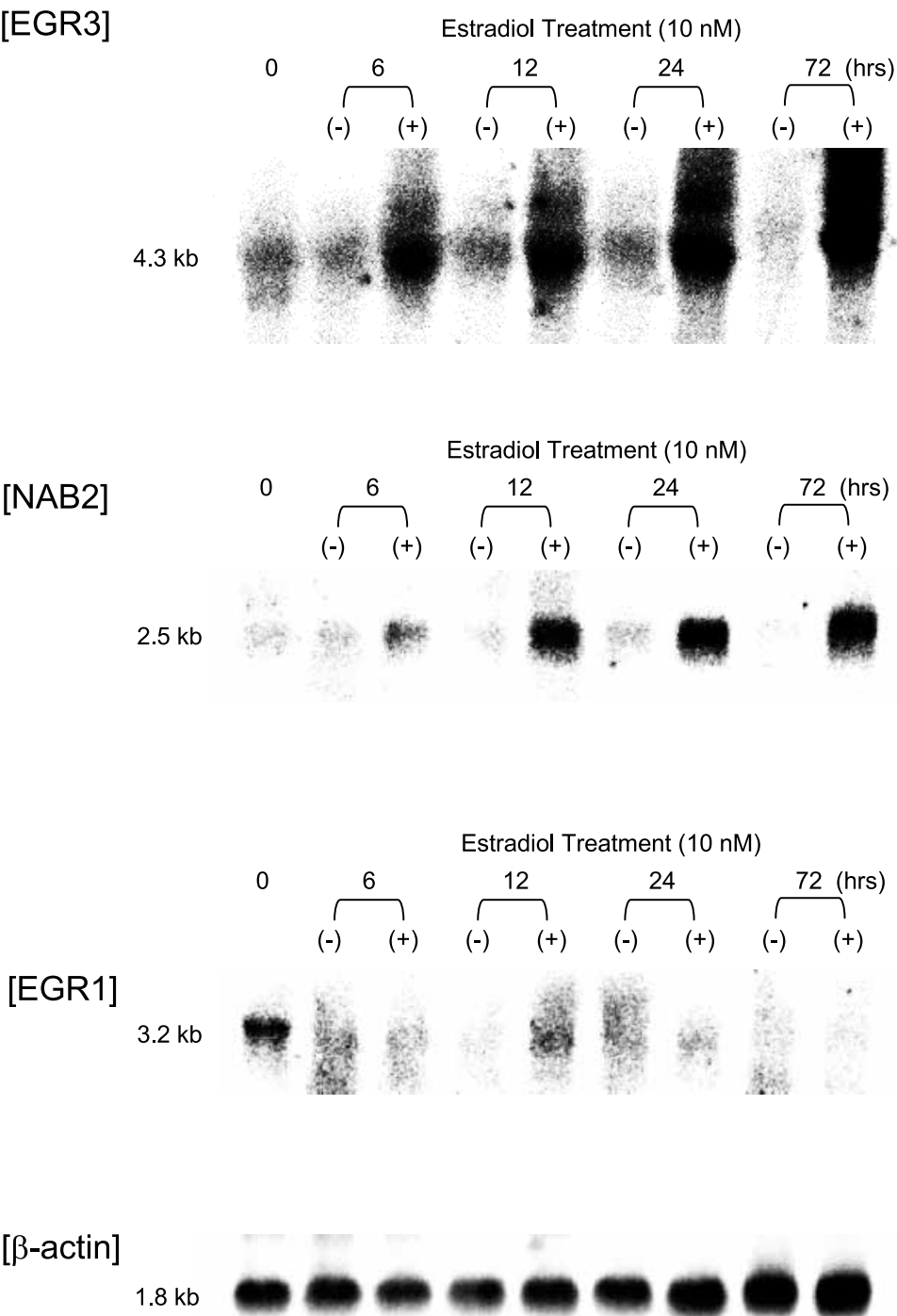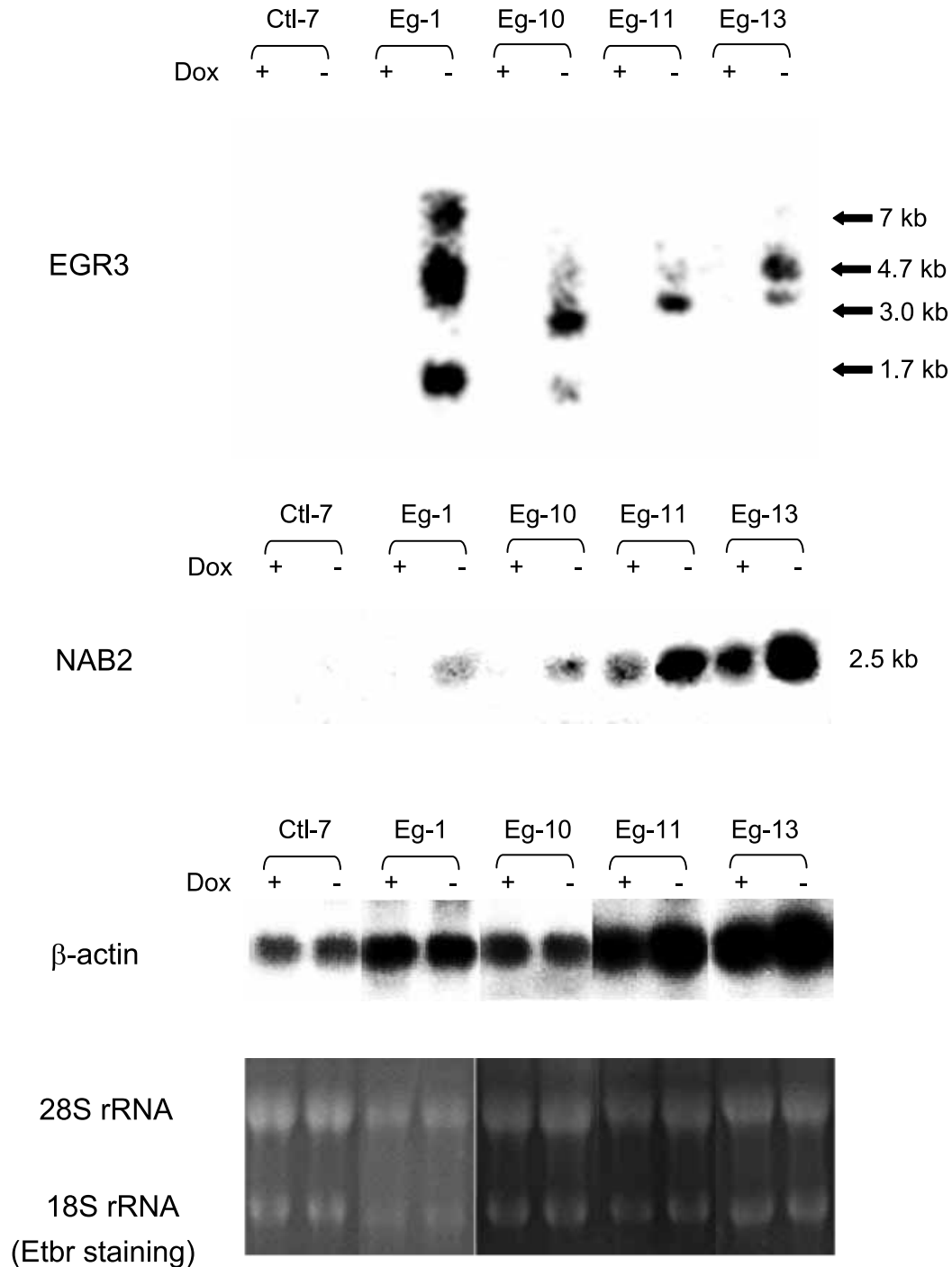
Figure 2 Northern blot analysis of the expression of *Egr3, Egr1* and *Nab2*.
Estrogen-starved MCF-7 cells were treated with 17β-estradiol or ethanol for 6, 12, 24,
and 72 h, and total RNA was isolated from each cell culture and from the cells without
any treatment (shown as 0 h). From each sample, 20 µg total RNA were taken for
Northern blot analysis, and cDNA fragments of the genes listed on the left were used as
probes. The sizes of the transcripts are also shown on the left of the detected transcripts.
Northern blot analysis of β-actin was also carried out as an internal control.
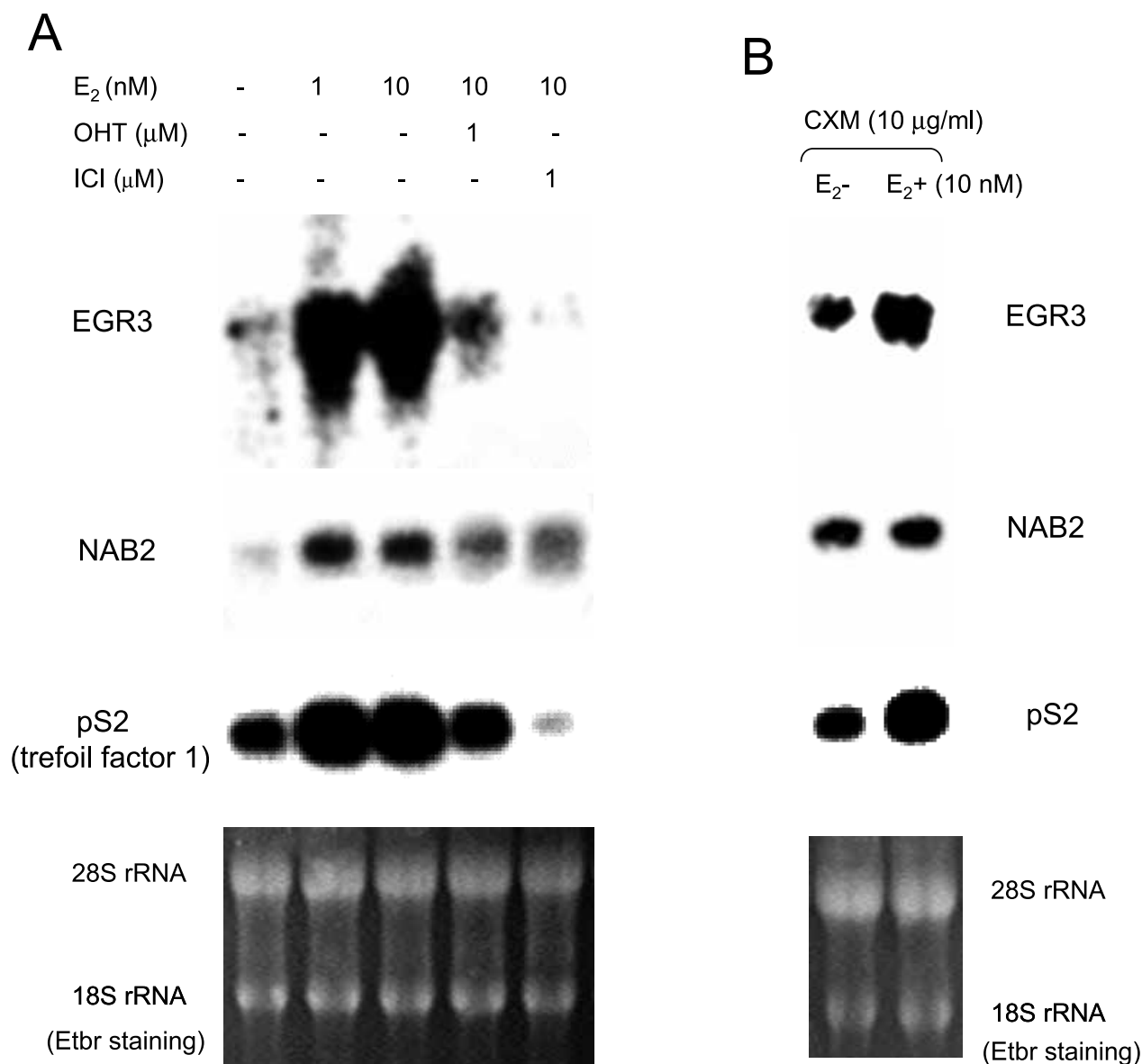
Figure 3 Northern blot analysis to examine the effects of antiestrogens and cycloheximide. (A) MCF-7 cells grown in estrogen-starved culture were treated with 17β-estradiol (E$_2$) alone, or E$_2$ and either 4-hydroxytamoxifen (OHT) or ICI 182,780 at the indicated concentrations. After treatment for 72 h, total RNA was isolated from the cells, and Northern blot analysis was performed for *Egr3*, *Nab2* and *pS2*. (B) Estrogen-starved MCF-7 cells were treated with ethanol or 17β-estradiol together with cycloheximide (CXM) for 18 h. Northern blot analysis was performed as for panel A. In both panels, gels stained with ethidium bromide (Etbr) are shown at the bottom.

was upregulated by estrogen in MCF-7 cells, and by the removal of doxycycline (by induction of *Egr3*) in one of the transformants (Fig. 5, cluster A and B). For example, a gene encoding TSC403 protein showed a 2·5-fold induction by estrogen in parental MCF-7 cells and 2·3-fold upregulation by removal of doxycycline in the clone Eg-11. One gene, which

is suggested to be similar to chemokine receptor 2, exhibited 1·9- and 2·1-fold upregulation in parental and Eg-11 cells respectively. The proto-oncogene *c-fos* was induced 2·5-fold and 2·1-fold in parental and Eg-10 cells respectively. These genes may be target genes induced by EGR3. On the other hand, in another clone, Eg-1, almost all the genes loaded

Figure 4 Northern blot analysis of *Egr3* and *Nab2* expression in stable transformants derived from MCF-7 cells. Transformants stably transfected with empty vector (Ctl-7) or *Egr3*-expression vector (Eg-1, -10, -11 and -13) were grown for 2 days in the presence (Dox+) or absence (Dox−) of 50 ng/ml doxycycline. Northern blot analysis for *Egr3* and *Nab2* was performed as in Figs 2 and 3. Northern blot analysis of β-actin was also carried out as an internal control. Gels stained with ethidium bromide (Etbr) are shown at the bottom.
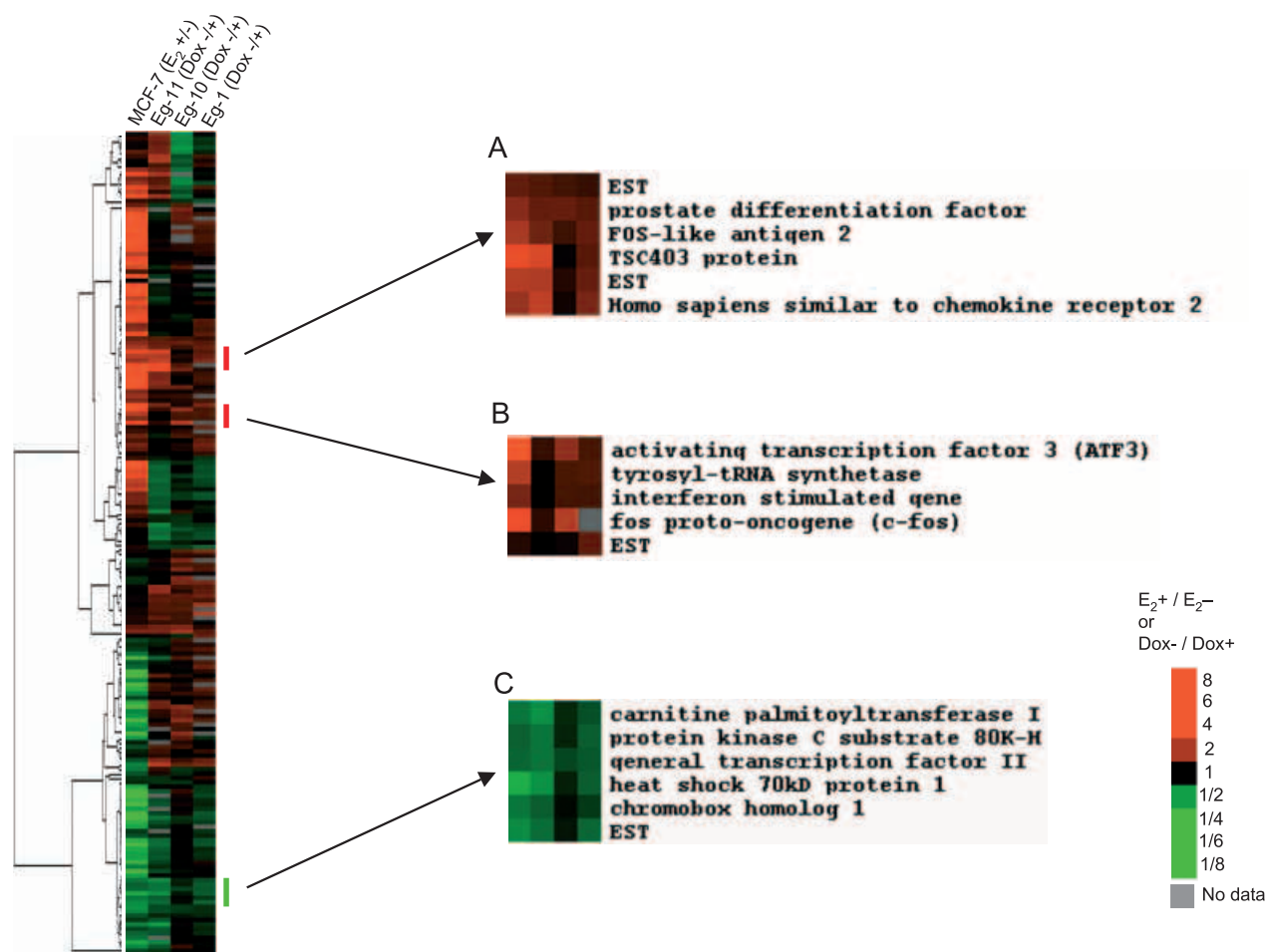
Figure 5 Gene-expression profiles of MCF-7 cells with EstrArray. *Egr3*-expressing Eg-1, -10 and -11 clones grown in estrogen-deprived media were cultured for 72 h in the presence (Dox+) or absence (Dox−) of 100 ng/ml doxycycline, and poly (A)+ RNA was isolated from the cells. Hybridization into EstrArray was performed as described previously (Inoue *et al.* 2002) and according to the manufacturer's instructions. Estrogen-responsive gene-expression profiling of parental MCF-7 cells was also performed with EstrArray, and the results are shown for comparison. After filtrating of the data of expression ratios, as described previously (Inoue *et al.* 2002), 186 genes for which there were remaining data in at least three cell lines were selected from the total of the genes loaded on EstrArray, and analyzed by hierarchic clustering. The results are represented as a pseudocolor visualization matrix. The characters of gene clusters denoted by the bars and letters A to C are as follows: (A) a group containing the genes whose differential expression ratios are nearly or more than 2·0, both in parental MCF-7 cells and clone Eg-11; (B) a group containing a gene (*c-fos*) whose differential expression ratio is equal to or more than 2·0, both in parental MCF-7 cells and clone Eg-10; (C) a group including the genes repressed by estrogen and removal of doxycycline in parental MCF-7 cells and clone Eg-11 respectively. All the gene groups are zoomed, and the expression patterns with gene names are shown on the right. The color scale used to represent the expression ratios is shown on the lower right. The gray color means that there are no reliable data after filtrating due to poor hybridization.

on EstrArray showed no significant induction by removal of doxycycline. Among the estrogen-repressed genes, some genes had similar expression patterns between parental MCF-7 cells and Eg-11 cells (Cluster C), but the rationale of repression of these genes by EGR3 is unknown.

Promoter of *fasL* was activated by EGR3 in breast cancer cells

The *fasL* gene is the only gene so far identified as a target of EGR3. It was demonstrated that, in activated T cells, induction of *fasL* mRNA is
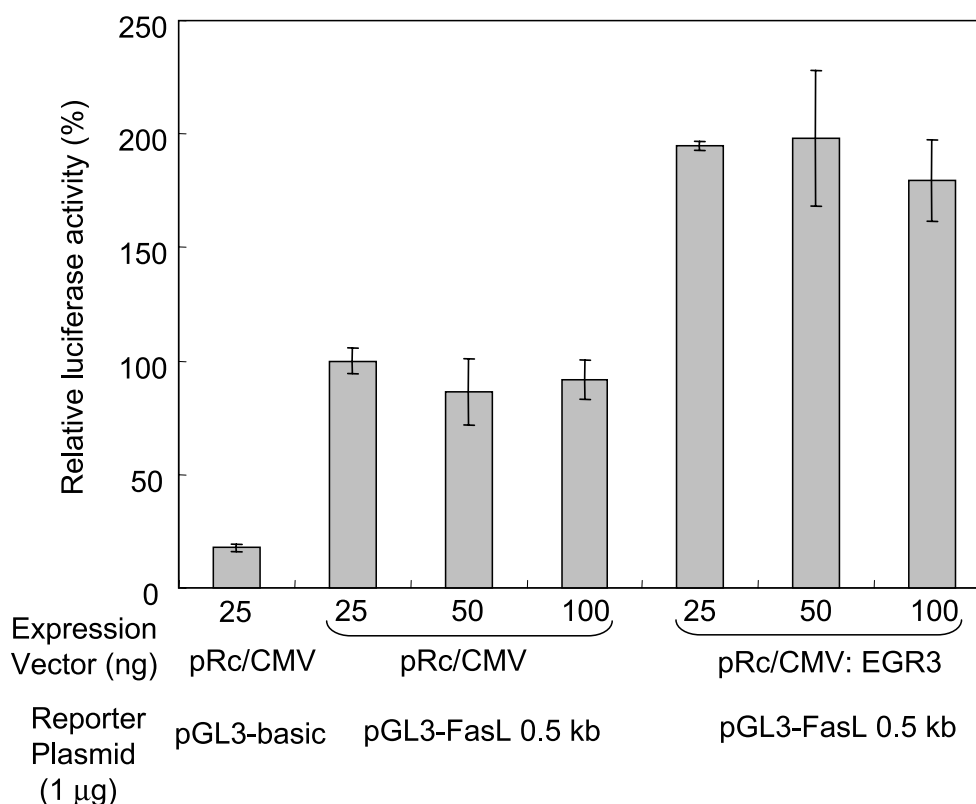
Figure 6 Luciferase reporter assay for the activity of *fasL* promoter. Luciferase reporter plasmid harboring a 0·5 kb fragment upstream of *fasL* was transfected into estrogen-starved MCF-7 cells together with the *Egr3*-expression vector at the indicated dosage. A reporter plasmid without the promoter fragment (pGL3-basic) and empty expression vector (pRc/CMV) were also used for controls. After incubation for 30 h, the luciferase activity of the cell lysate was measured and normalized to the internal control luciferase activity. The results are shown as the average±standard deviation (*n*=3) of relative luciferase activity where that of the cells transfected with 25 ng pRc/CMV and 1 μg pGL3-FasL 0·5 kb was taken as 100%.

mediated by EGR3, and the 16-mer sequence called Fas ligand regulatory element (FLRE) in the promoter region is sufficient for *fasL* expression induced by EGR3 (Mittelstadt & Ashwell 1998). It was also reported that a reporter plasmid bearing the 16-mer FLRE was also transactivated by ectopically expressed EGR3 in nonlymphoid HeLa cells (Mittelstadt & Ashwell 1998), implying that the regulation of *fasL* expression by EGR3 may be a common pathway existing not only in lymphoid cells but also in other tissues. We at first tried to examine the expression levels of *fasL* mRNA in *Egr3*-expressing transformants and parental MCF-7 cells. However, quantitative analysis was impossible, while *fasL* mRNA could faintly be detected in parental MCF-7 cells (data not shown), presumably

because of too low expression levels or instability of *fasL* mRNA. EstrArray is also unavailable for analysis of *fasL* because *fasL* cDNA is not loaded on it. Therefore, we examined the *fasL* expression using a reporter assay. Luciferase reporter vector harboring a 0·5 kb upstream region of the *fasL* gene containing FLRE was transiently transfected into MCF-7 cells together with *Egr3* expression vector containing a constitutive CMV promoter upstream of *Egr3*, and the activity of the *fasL* promoter was examined. As shown in Fig. 6, overexpression of *Egr3* increased *fasL* promoter activity approximately twofold compared with the case without ectopic expression of *Egr3*. This observation suggests that *fasL* may be another target gene for EGR3 in breast cancer as well as in T cells.

Discussion

In this study, we focused on the functional analysis of *Egr3* encoding a zinc-finger transcription factor in breast cancer cells. We have previously identified *Egr3* as one of the estrogen-responsive genes that was induced in breast cancer MCF-7 cells after estrogen treatment for 72 h. Estrogen-dependent induction of this gene was also observed in cell lines derived from various other tissues. Northern blot analysis in this study revealed that *Egr3* was induced by estrogen at the early phase (at the latest within 6 h after estradiol treatment) independently of *de novo* protein synthesis, and antiestrogens blocked the inducible expression of *Egr3*. These observations strongly suggest that *Egr3* is one of the primary targets for the ER. There are at least three EREs (estrogen-responsive elements) upstream of *Egr3* (−2843 to −2839, −2701 to −2697 and −693 to −689 bp from the transcriptional initiation site) to which the ER would bind, leading to transactivation. However, it is unknown and requires further investigation whether these elements are responsible for ER-mediated transactivation of the gene.

Northern blot analysis of *Nab2* in *Egr3*-overexpressing stable transformants suggested that *Nab2* is one of the downstream target genes for EGR3. This conclusion is consistent with the results from Northern blot analysis using parental MCF-7 cells, in which *Nab2* was also induced by estrogen, but this was dependent on *de novo* protein synthesis. NAB2 was at first identified as a corepressor for EGR1 (Svaren *et al.* 1996), but in some cases it was demonstrated to work as a coactivator for EGR3-mediated transactivation (Sevetson *et al.* 2000). NAB2 is considered to exert either positive or negative effects on the transactivation of target genes for EGR3, depending on the promoter construction of the genes. In either case, it is reasonable to assume that estrogen-induced EGR3 in turn enhances the expression of NAB2, an effect which may have feedback effects on expression patterns of EGR3-induced genes at a later phase in breast cancer cells exposed to estrogen.

Gene-expression profiling in *Egr3*-expressing clones using EstrArray found other candidate target genes induced by EGR3. Among them, the TSC403 gene has been reported to encode protein similar to lysosomal membrane glycoproteins (lamps) 1 and 2, and demonstrated to be overexpressed only in malignant cells, but not normal cells, in several tissues such as the breast and colon (Ozaki *et al.* 1998). TSC403 may be involved in the genesis and/or progression of breast tumors. The proto-oncogene *c-fos* encoding a transcription factor has been demonstrated to be induced by estrogen at an early phase in MCF-7 cells (Duan *et al.* 1999). This gene is considered to be one of the primary targets for ER, but another pathway might exist for transactivation of *c-fos* at a late phase mediated by EGR3. It is unknown why different gene-expression patterns were observed in the three transformants, although regulation of *Egr3* expression by doxycycline was prominent in all the clones (Fig. 4). It is possible that the expression patterns of target genes for EGR3 may be influenced not only by the amounts of EGR3 proteins but also by the relative amounts of cofactor NAB2 induced by EGR3, because there were quite different *Nab2* expression levels in these clones.

On the other hand, *fasL* is the only target gene for EGR3 so far identified. A limited region upstream of the *fasL* gene has been demonstrated to be sufficient for EGR3-mediated transactivation, both in T cells and nonlymphoid HeLa cells, a fact which prompted us to test whether the *fasL* promoter could also be activated by EGR3 in breast cancer cells. The luciferase reporter assay suggested that *fasL* is another target gene for EGR3 in breast cancer. There are several reports concerning the expression of Fas ligands in tumors derived from several organs such as the breast (Gutierrez *et al.* 1999) and ovary (Rabinowich *et al.* 1998). FasL expression in these malignant cells is considered to be one of the mechanisms involved in escape from immune surveillance, leading encountered T cells into apoptosis, and it would bring about an advantage in the progress and metastasis of the tumor cells *in vivo*. Considering these facts, it can be hypothesized that EGR3 has an important role in the estrogen-dependent induction of the immune evasion system in ER-positive breast cancer, although further study will be needed to demonstrate this attractive hypothesis.

In conclusion, we believe that identification of *Egr3* as one of the ER-mediated estrogen-induced genes sheds light on a novel estrogen-signaling pathway in breast cancer cells. We hypothesize the scheme and role of the novel estrogen-signaling pathway as shown in Fig. 7. In this pathway,
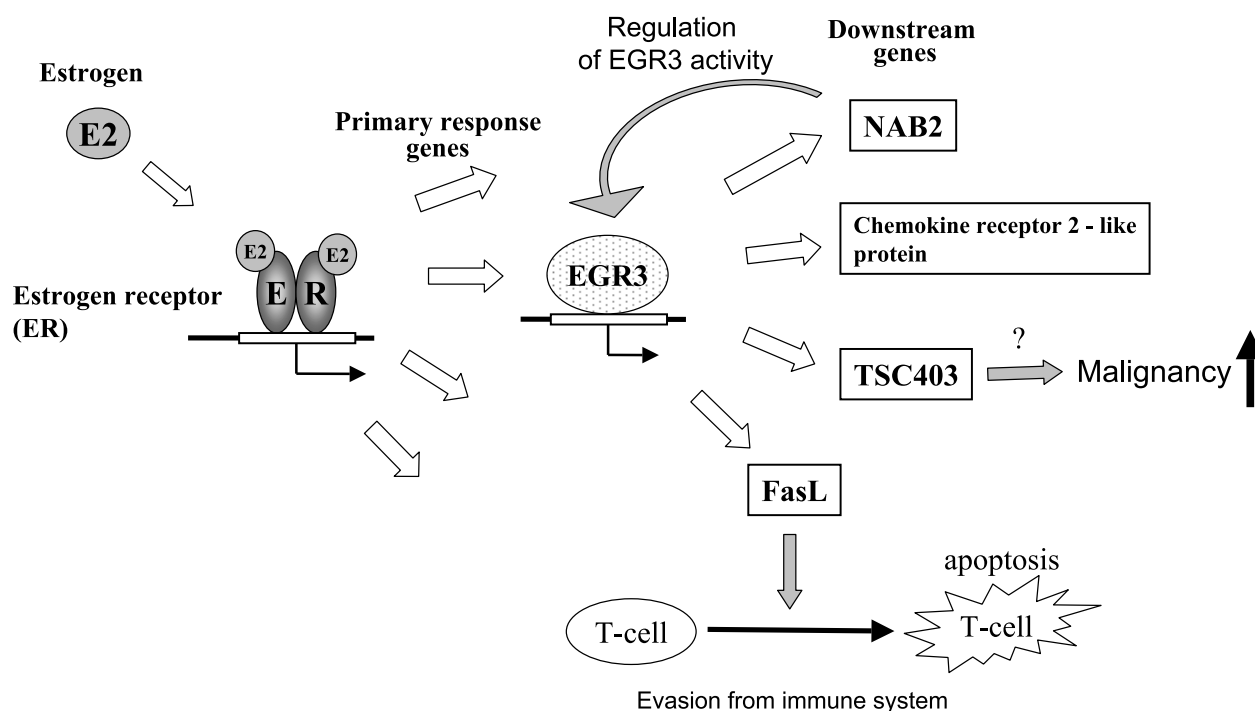
Figure 7 Hypothetical schema of EGR3-mediated estrogen-signaling pathway in MCF-7 cells.

estrogen and ER induce the expression of *Egr3*, and EGR3 protein in turn induces a specific cofactor, NAB2. EGR3 also induces other genes such as the TSC403 gene and *fasL*, with its activity being modulated by NAB2. Considering previous reports on them, these gene products may have no effect on the growth of breast cancer. However, these proteins may play their own roles in conferring superiority in the progression and metastasis of breast tumors which have emerged *in vivo*.

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