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

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The biological roles of estrogen-responsive finger protein (efp) *in vivo* were evaluated in mice carrying a loss-of-function mutation in efp by gene-targeted mutagenesis. Although efp homozygous mice were viable and fertile in both sexes, the uterus that expressed abundant estrogen receptor α exhibited significant underdevelopment. When the ovariectomized homozygotes were subjected to 17β-estradiol treatment, they showed remarkably attenuated responses to estrogen, as exemplified by decreased interstitial water imbibition and retarded endometrial cell increase, at least, attributable to the lower ratio of G1 to S-phase progression in epithelial cells. These results suggest that efp is essential for the normal estrogen-induced cell proliferation and uterine swelling as one of the direct targets of estrogen receptor α.

It is known that estrogen is not only essential in the reproductive system as a sex steroid hormone but also effective to protect postmenopausal women from osteoporosis, coronary heart disease, and Alzheimer disease (1, 2). Estrogen receptors (ERs) such as ERα and ERβ (3–6) act as estrogen-dependent transcription factors by binding to specific estrogen-responsive elements (ERE) in the enhancer region of target genes and regulating their transcription directly (7, 8). The ligand-dependent activation of ERs is accompanied by an induction of binding of coactivator proteins that facilitate functional interaction of the receptors with the general transcription machinery (9–11). Although it is generally believed that estrogen could exert its action on target organs by regulating target gene products, relatively few target genes with consensus ERE are known so far, including vitellogenin (12), ovalbumin (13), lactoferrin (14), prolactin (15), progesterone receptor (16), cathepsin D1 (17), and pS2 (18). Just how these genes actually mediate which part of estrogen action *in vivo* has been quite difficult to evaluate. Besides, molecular mechanisms of estrogen action are more complicated by additional cascade via factors such as AP-1 (19). We have therefore been trying to obtain more downstream target genes of ERα by using a method named genomic binding-site cloning in which genomic ERE-containing DNA fragments are isolated by binding with the DNA binding domain of the recombinant ERα protein (20–22).

We have thus cloned from a human cDNA library several estrogen-regulated genes, one of which was designated the estrogen-responsive finger protein (efp) gene (23–25). Both human and mouse efp cDNAs possess a palindromic ERE in 3′ noncoding region, and the proteins have a RING finger domain at the N terminus that might be involved in the protein–protein interaction. These proteins also have a pair of zinc fingers and a coiled-coil region in the 3′-terminal side of the RING finger domain, a structure resembling promyelocytic leukemia (26–29) and Midline 1 (30). Incidentally, BRCA1 (31), which is the suppressor of a type of familial mammary carcinoma, also has the RING finger, but not the zinc fingers. The mouse efp mRNA was abundantly expressed in the uterus, the ovary, and the placenta. Moreover, the efp mRNA was rapidly up-regulated in the uterus by 17β-estradiol injection, and the colocalized expression with the ERα mRNA was shown in the uterine epithelial cells, the ovarian granulosa cells, and the mammary gland epithelial cells (24). These findings suggested the possibility that efp might mediate estrogen action in various female organs (24). To clarify biological functions of efp in relation to estrogen action, we have here generated mice in which the efp gene is disrupted and have characterized their phenotype.

Materials and Methods

efp Gene Targeting Strategy and Generation of Knockout Mice. A mouse genomic clone was isolated by screening the 129 SVJ Lambda Fix II library (Stratagene) using an ≈520-bp fragment of the mouse efp cDNA (24) including exon 1. The targeting vector was constructed by using mouse genomic clones and contained a PGK-neo cassette (32) for positive selection and diphtheria toxin A (33) for negative selection against random integration. It contained 8.0 and 2.0 kilobases (kb) of 5′ and 3′ homologous genomic sequences, respectively, surrounding the PGK-neo cassette (Fig. 1*A*), resulting in most of exon 1, including the RING finger domain, being deleted. J1 embryonic stem (ES) cells (34) were electroporated with 25 μg of the targeting vector linearized at a unique *Not*I site and subjected to positive selection using G418. After the selection, 423 resistant ES cell clones were subcloned, and 5 independent targeted clones were identified by Southern blot analysis. Four mutant clones were injected into C57 BL/6 recipient blastocysts to produce male chimeras, and these males were crossed with 129SV female. Germ-line transmission of efp mutant allele was obtained for all four targeted clones. Heterozygous F1 animals from each clone were intercrossed to produce homozygous mutants. Genotyping of tail DNA in F2 offspring also was performed by Southern blots and PCR analyses with the primer 1 (5′-ATGGCGGAGCTGAATCC-3′) and the primer 2 (5′-GTGCTGGGTACATTTGCG-3′) in exon 1 and the primer 3 (5′-TAGCCTGAAGAACGAGATC-3′) in the PGK-neo cassette.

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Abbreviations: efp, estrogen-responsive finger protein; ERα, estrogen receptor α; ERE, estrogen-responsive elements; kb, kilobase; ES cell, embryonic stem cell.

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Animals and Injections Schedule for Estimation of Estrogen Responsiveness. Adult F2 females were ovariectomized on day 0 and were treated with 17β-estradiol (E2) (20 μg/kg/day) or olive oil (solvent control) from day 28 to day 30, and each uterine wet weight from E2-treated wild-type mice (+/+) (n = 11), heterozygotes (+/−)(n = 11), homozygotes (−/−) (n = 11), and non-E2-treated mice was measured on day 31. The ratio of wet weight (milligrams) to body weight (grams) was calculated. Then, frozen sections from each specimen were generated for histological analysis. Subsequently, to monitor the fluid uptake, adult F2 females were ovariectomized on day 0 and were treated with E2 (20 μg/kg/day) or olive oil (solvent control) from day 28 to day 30 as described above, and then uterine water imbibition and dry weight from E2-treated wild-type mice (+/+) (n = 6), homozygotes (−/−) (n = 6), and non-E2-treated mice were measured on day 31. The uteri from animals were slit longitudinally and were blotted to remove luminal fluid, and then the organs were incubated in a 60°C oven for 1 day and were weighed finally. The difference between wet and dry weight allowed for determination of the water imbibition, and the ratio of water imbibition and dry weight (milligrams) of the uterus to body weight (grams) was calculated, respectively.

Animals and Injections Schedule for BrdUrd Labeling Experiment. Adult F2 females were ovariectomized on day 0 and were treated with E2 (2 μg/kg) or olive oil (solvent control) for 12 h on day 28. The animals received an intraperitoneal injection of BrdUrdrd (30 mg/kg) 2 h before being killed. *In vivo* BrdUrd labeling was performed by intraperitoneal injection of BrdUrd as described (35). Then, each uterus from E2-treated wild-type mice (+/+) (n = 8), homozygotes (−/−) (n = 8), and non-E2-treated mice was transversely separated into three portions. Subsequently, three respective frozen sections from the uterus were immunostained with anti-BrdUrd monoclonal antibody, and an average of the BrdUrd-positive epithelial cell counts in them was calculated. The BrdUrd labeling index was finally calculated as the percentage of scored BrdUrd-positive epithelial cells in whole epithelial cells stained by 4′,6′ diamino-2-phenylindole, and at least 400 cells were scored.

Northern Blot Analysis. For each sample, 5 μg of Poly(A)$^+$RNAs from the uterus of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice were separated in 1% agarose. Northern blot analysis was performed as described (36). The ^{32}P-labeled 1.3-kb *Eco*RI-*Xho*I fragment, including most the coiled-coil and the C-terminal region but not the RING finger domain of the mouse efp cDNA (24) or the glyceraldehyde-3-phosphate dehydrogenase cDNA, was used as a probe. Autoradiography was carried out at −80°C with an intensifying screen for 3 days with the mouse efp cDNA probe and for 1 day with the glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

Preparation of Nuclear Extracts and Western Blot Analysis. Nuclear extracts were prepared from the uterus of a wild type (+/+), a heterozygote (+/−), and a homozygote (−/−) as described (37). Thirty micrograms of nuclear extract was resolved by electrophoresis on a 15% SDS polyacrylamide gel. The immunoblotted membrane was reacted with anti-mouse efp antibody (24) (1:5,000), was treated with anti-rabbit IgG (Fc) conjugated with horseradish peroxidase (HRP) (1:7,500), and then was detected by using ECL detection reagents (Amersham Pharmacia).

Immunohistochemistry. Immunohistochemistry was performed as described (36). Frozen sections of the uterus from an 8-week-old ICR mouse were reacted by using anti-mouse efp antibody (24). Subsequently, biotinylated anti-rabbit IgG antibody was reacted, and horseradish peroxidase (HRT)-streptavidin and substrate solution were added. Then sections were slightly stained by hematoxylin. In addition, for evaluation of the BrdUrd labeling index, frozen sections of the uterus from each genotype (+/+, −/−) treated with BrdUrd were generated, and then immunofluorescence using anti-BrdUrd monoclonal antibody (Caltag, South San Francisco, CA) was performed as described (35). They were stained by anti-mouse IgG (Jackson ImmunoResearch) with FITC and were embedded in 25% glycerol with 4′,6′ diamino-2-phenylindole (Sigma).

Results

The strategy for targeted deletion of the endogenous mouse efp gene is outlined in Fig. 1*A*. Because exon 1 of efp encodes the translation start site and the RING finger domain, we sought to disrupt exon 1 of this gene. The operation replaced a large portion of exon 1 except for the 17 amino acids from the translation start site with a PGK-neo cassette (32) in reverse orientation (Fig. 1*A*). J1 ES cells (34) were electroporated with the targeting vector and were selected by using G418. Five independent targeted ES cell clones obtained by homologous recombination were identified by Southern blot analysis using the probe a and the probe neo (Fig. 1 *A* and *B*). Germ-line transmission of the mutated efp allele was accomplished for four independent targeted clones, and heterozygous breeding was set up for F1 animals derived from each clone to produce homozygous mutants. The genotype of F2 offspring was determined by Southern blots (Fig. 1*C 1*, *2*, and *3*) and PCR analyses (Fig. 1*C4*), and the results showed a ratio of efp genotypes close to 1:2:1 expected for Mendelian inheritance. The efp mRNA was not detected in the uterus of a homozygous mutant mouse by Northern blot analysis of the Poly(A)$^+$RNAs whereas the transcript was clearly detected in a wild-type mouse (Fig. 1*D*). To confirm the absence of the efp protein in a homozygote, we performed immunoblotting of extracts prepared from the uterus by using a polyclonal antibody (24) specific to the region of amino acids 305–458 of the mouse efp protein. The efp protein was undetectable in the uterus of a homozygote and was almost half of the amount in a heterozygote compared with a wild-type mouse (Fig. 1*E*). On immunostaining, the efp protein was detected abundantly in the uterine epithelium of wild-type mice (Fig. 1 *F Upper*) but not of homozygotes (Fig. 1*F Lower*).

efp homozygous mutants appeared to grow normally, showing no obvious external defect in phenotype. They were even fertile for both sexes. We therefore examined target organs for estrogen in F2 animals with pure 129 SV genetic background. First, we inspected the uterine horn size and the weight for 10-week-old F2 offspring. A number of uteri at the secretory phase of menstrual cycle were collected from F2 offspring by checking the vaginal smear. The uteri of homozygous mice were significantly smaller (Fig. 2*A*), showing the ratio of uterine weight to body weight 36% lower than that of wild-type mice (Fig. 2*B*), although there was no significant difference in the whole body weight between them. However, no histological abnormality was detected by hematoxylin and eosin staining in uterine cells of homozygous mice (data not shown). The uteri in heterozygotes also tended to be smaller, but the difference from the wild-type mice was not statistically significant (Fig. 2*B*). The possibility exists that haploinsufficiency of efp affects uterine development to some extent. In contrast, the weight of the liver in F2 animals was approximately the same (data not shown).

Next, we examined the estrogen-induced uterine growth after ovariectomy in efp homozygous mutants. Ovariectomized F2 animals were treated with a dose of E2 (20μg/kg/day). Wild-type and heterozygous mice responded well to E2, with an ≈7.3-fold increase in the ratio of uterine wet weight to body weight, compared with that of non-E2-treated wild-type and heterozygous mice, respectively (Fig. 3, *1*). In contrast, the ratio in E2-treated homozygotes showed a significantly lower increase, i.e., ≈5.4-fold compared with that of non-E2-treated homozygotes (Fig. 3, *1*). This average

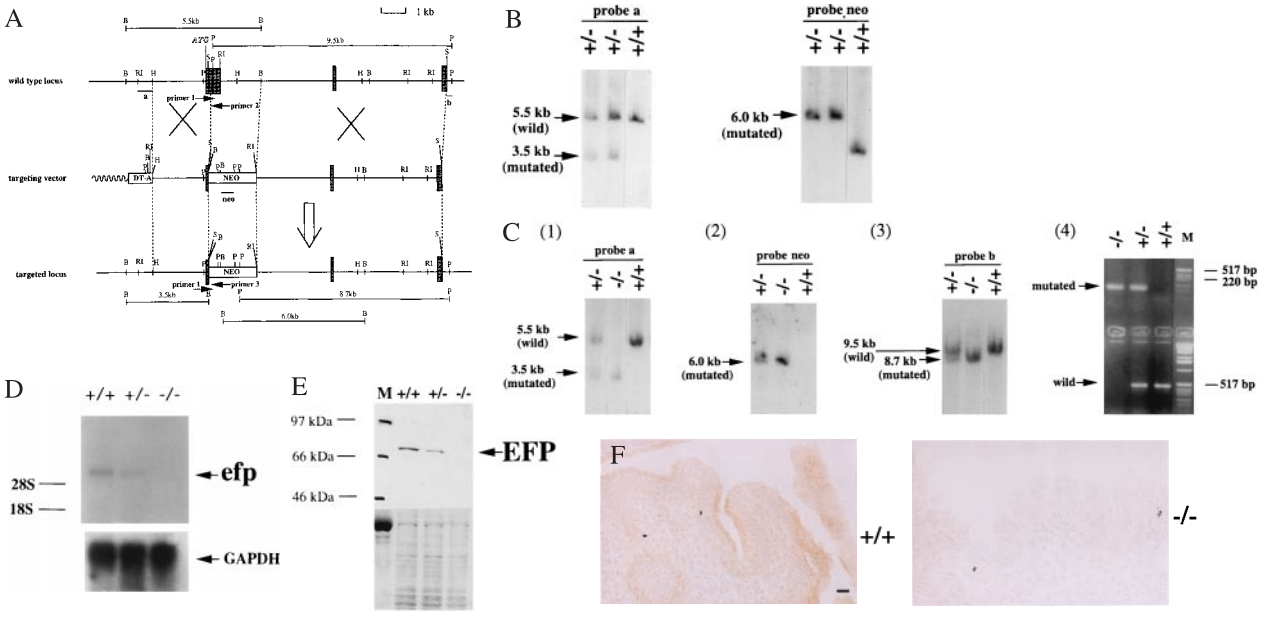

Fig. 1. Targeted disruption of the mouse efp locus. (*A*) Diagram of the mouse efp gene locus (*Top*), targeting construct (*Middle*), and resulting the efp gene targeted locus (*Bottom*). The wild-type locus of the efp gene (*Top*) contains three exons shown as a stippled box and ATG indicated into exon 1 codes predicted the first methionine. The replacement-type targeting vector (*Middle*) was used for electroporation. A PGK-neo-pA cassette (32), including the phosphoglycerate kinase promoter and polyadenylation signals, was inserted into the coding region of efp in reverse orientation, and diphtheria toxin A cassette (33) also was used. Three probes (a, neo, b) for Southern blot analysis and the expected size of the detected DNA fragment are indicated. Positions and orientations of primers used for genotyping wild type and mutated alleles by PCR analysis are designated by arrows (primer 1–3). B, *Bam*HI; RI, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I. (*B*) Identification of homologous recombinant ES cell clones. Genomic DNA extracted from two positive clones and one negative clone was digested by *Bam*HI and was analyzed by Southern blot analysis with the probe a, which located in the 5′ side of the targeting vector, and the probe neo. The result using the probe a reveals expected wild-type and mutated alleles of 5.5- and 3.5-kb fragments, respectively. The result using the probe neo reveals the expected mutated allele of a 6.0-kb fragment in homologous recombinant ES cell clones (+/−) but not in the negative control clone (+/+). (*C*) Southern blot and PCR analyses of the efp mutant allele in F2 offspring. Tail DNA from 3-week-old F2 offspring was isolated. Southern blot analysis was performed after *Bam*HI digestion (*C 1* and *2*) by using the probe a and the probe neo and after *Pst*I digestion (*C3*) by using the probe b. The results show wild-type and mutated alleles of 5.5- and 3.5-kb fragments, respectively, in the probe a, the only mutated allele of a 6.0-kb fragment in the probe neo, and the wild-type and mutated alleles of 9.5- and 8.7-kb fragments, respectively, in the probe b. Positive bands with expected sizes are observed in lanes of wild type (+/+), heterozygous (+/−), and homozygous (−/−) mice. Representative PCR analysis (*C4*) of genomic DNA from mouse tail of F2 offspring was performed by using the primer 1 and the primer 2 to detect the wild-type allele and by using the primer 1 and the primer 3 to detect the mutated allele. The genotypes and their corresponding PCR products are as follows; wild type (+/+), 475 bp; heterozygous (+/−), 475 and 152 bp; and homozygous (−/−), 152 bp. (*D*) Northern blot analysis of RNA extracted from the uterus of F2 offspring. The band of efp transcript is detected in a wild-type mouse (+/+) and a heterozygote (+/−) but is absence in a homozygote (−/−) whereas equivalent signals are detected for each by hybridization with the glyceraldehyde-3-phosphate dehydrogenase cDNA. (*E*) Protein immunoblot analysis. Nuclear extract (30 μg of each) from the uterus was resolved by electrophoresis on 15% SDS polyacrylamide gel and was immunoblotted with anti-mouse efp antibody (24). A band of efp at ≈70 kDa is shown in the extract of a wild-type mouse (+/+) and almost half of the amount in a heterozygote (+/−) but none in a homozygote (−/−). The staining with Coomassie brilliant blue is also shown below, showing that equivalent extracts were used. M, molecular marker. (*F*) Immunohistochemical localization of the mouse efp protein in uterine epithelial cells. The positive staining over uterine epithelial cells in a wild-type mouse (+/+) (*Upper*) indicates the mouse efp immunoreactivity. Note the absence of immunoreactivity in a homozygote (−/−) (*Lower*). (Bar = 100 μm.)

decrease of 26% was statistically significant ($P < 0.05$). Next, water imbibition and dry weight of the uterus in E2-treated homozygotes were compared with those of wild-type mice to evaluate the extent of the fluid uptake and the net increase of dry mass. The results

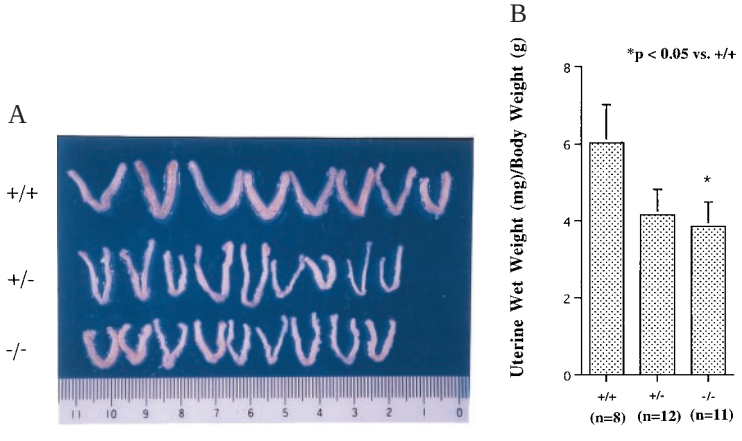

Fig. 2. Uterine growth and development in F2 offspring. (*A*) Estimation of size of uteri in 10-week-old F2 offspring. Several uteri with secretory phase in menstrual cycle were collected. Underdevelopment in homozygous mice (−/−) as compared with wild-type mice (+/+) is actually shown. (*B*) Evaluation of uterine development in F2 offspring. The ratio of uterine wet weight (milligrams) to body weight (grams) was calculated, and the ratio in homozygous mice (−/−) ($n = 11$) is lower than in wild-type mice by 36%. The *t* test shows a significant difference ($P < 0.05$) between wild-type mice (+/+) ($n = 8$) and homozygotes (−/−) ($n = 11$).

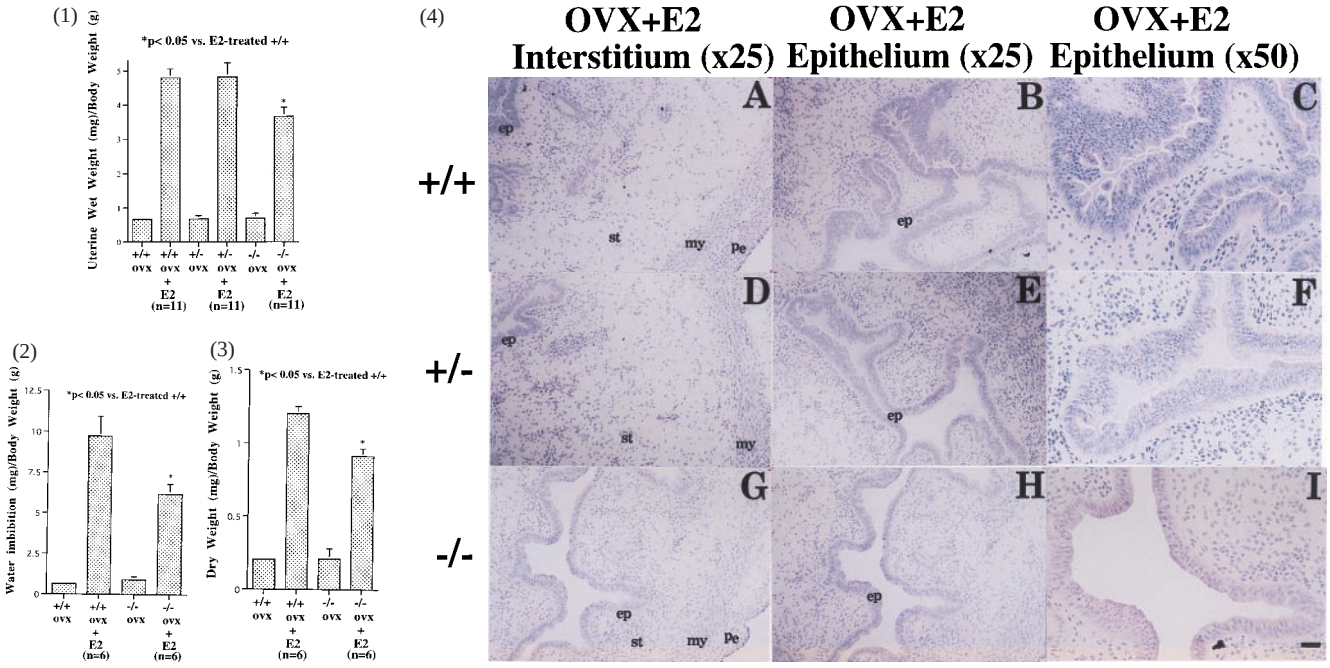

Fig. 3. Uterine response to estrogen stimulation in F2 animals. (*1*) The ratio of uterine wet weight (milligrams) to body weight (grams) was calculated. The increase between E2-treated homozygotes (−/−) and olive oil-treated homozygotes (−/−) is lower than the increase between E2-treated wild-type mice (+/+) and olive oil-treated wild-type mice (+/+) by an average of 26%. Olive oil was used as solvent control. The *t* test shows a significant difference ($P < 0.05$) in E2-treated wild-type mice (+/+) ($n = 11$) and E2-treated homozygotes (−/−) ($n = 11$). (*2, 3*) The ratio of uterine water imbibition and dry weight (milligrams) to body weight (grams) was calculated. The increase between E2-treated homozygotes (−/−) and olive oil-treated homozygotes (−/−) is lower than the increase between E2-treated wild-type mice (+/+) and olive oil-treated wild-type mice (+/+) by 54% in water imbibition (*2*) and by 22% in dry weight (*3*), respectively. Olive oil was used as solvent control. The *t* test shows a significant difference ($P < 0.05$) between E2-treated wild-type mice (+/+) ($n = 6$) and E2-treated homozygotes (−/−) ($n = 6$) in both water imbibition (*2*) and dry weight (*3*). (*4*) Histological analysis in the uterus of F2 animals after estrogen injection. The uterus from ovariectomized F2 animals with E2 (20 μg/kg/day) was collected and quickly frozen. Each horizontal section was stained by hematoxylin and eosin and was examined histologically. *A*, *B*, and *C* represent wild-type mice (+/+), *D*, *E*, and *F* heterozygotes (+/−), and *G*, *H*, and *I* homozygotes (−/−). The portion of the interstitium (×25) is shown in *A*, *D*, and *G*, that of the epithelium in *B*, *E*, and *H* (×25) and *C*, *F*, and *I* (×50). The decreased extent of water imbibition into the interstitium (*G*) and of hyperplasia in epithelial cells (*H* and *I*) is shown clearly in the uterus of E2-treated homozygote (−/−). ep, epithelium; st, stroma; my, myometrium; pe, perimetrium. [Bars = 100 μm (*C*, *F*, and *I*) and 200 μm (*A*, *B*, *D*, *E*, *G*, and *H*).]

indicated that the increases of both water imbibition and dry weight of the uterus were significantly impaired in homozygotes (Fig. 3, *2* and *3*), showing 54% lower increase (6.8-fold) in water imbibition (Fig. 3, *2*) and 22% lower increase (4.6-fold) in dry weight (Fig. 3, *3*), respectively. Incidentally, wild-type mice showed increases of 15.1-fold and 5.9-fold for water imbibition and for dry weight, respectively.

In addition, histological analysis of the uterus in E2-treated F2 mice whose wet weight was estimated in Fig. 3, *1* was carried out. The uteri from E2-treated wild-type and heterozygous mice showed enough response, as was expected, with respect to water imbibition into the interstitium and hyperplasia of epithelial cells (Fig. 3, *4 A–F*) accompanied by the invasion of eosinocyte (data not shown) whereas that from E2-treated homozygotes showed much decreased response in the extent of water imbibition into the interstitium (Fig. 3, *4G*) and of hyperplasia in epithelial cells (Fig. 3, *4 H* and *I*) but with rather normal invasion of eosinocyte.

To get further insight into the cause of poor uterine cell response to estrogen in efp homozygous mutants, we examined the responsiveness of homozygous mutant cells to estrogen with respect to cell cycle progression. We treated ovariectomized F2 offspring with E2 for 12 h and injected bromodeoxyuridine (BrdUrd) 2 h before killing them, and then the BrdUrd labeling of endometrial cells was examined by using immunostaining with anti-BrdUrd monoclonal antibody (see *Materials and Methods*). A 10.7-fold increase in the BrdUrd labeling index was observed in the tissues of wild-type mice treated with E2 (Fig. 4 *Upper and Lower*, *C*), as compared with non-E2-treated wild-type mice (Fig. 4 *Upper and Lower*, *A*) whereas only a 4.4-fold increase of the BrdUrd labeling index was noted in uterine epithelial cells of E2-treated homozygotes (Fig. 4 *Upper and Lower*, *E*) compared with non-E2-treated homozygotes, indicating that smaller population (≈41%) of the epithelial cells entered the S phase by E2 stimulation in homozygotes than in wild-type mice.

Discussion

Estrogen receptors, ERα and ERβ, are critical mediators of estrogen action in target tissues. The phenotypes of the severe hypoplastic uterus with quite a low response to estrogen and the enlarged and cyctic dysfunctional ovary found in infertile ERα-deficient mice (38, 39) clearly show that ERα is a prerequisite for the development of female organs such as the uterus and the ovary. A recent paper on the ERβ knockout mice (40) has reported phenotypes of ovarian dysfunction such as earlier arrest in folliculogenesis and disorder of ovulation but not uterine abnormality. This demonstrates that ERα has a more important role in uterine function than ERβ does. It also has been supposed from more abundant expression of ERα than ERβ that the uterine cell proliferation and remarkable water imbibition induced by estrogen administration would be chiefly mediated by ERα. In addition, recent reports on aromatase-deficient mice (41, 42) show that the depletion of estrogen also caused underdevelopment of the uterus. However, the molecular signaling leading to cell proliferation and the growth regulated by estrogen-dependent activation of ERα is yet enigmatic in the uterus as well as in other target organs.

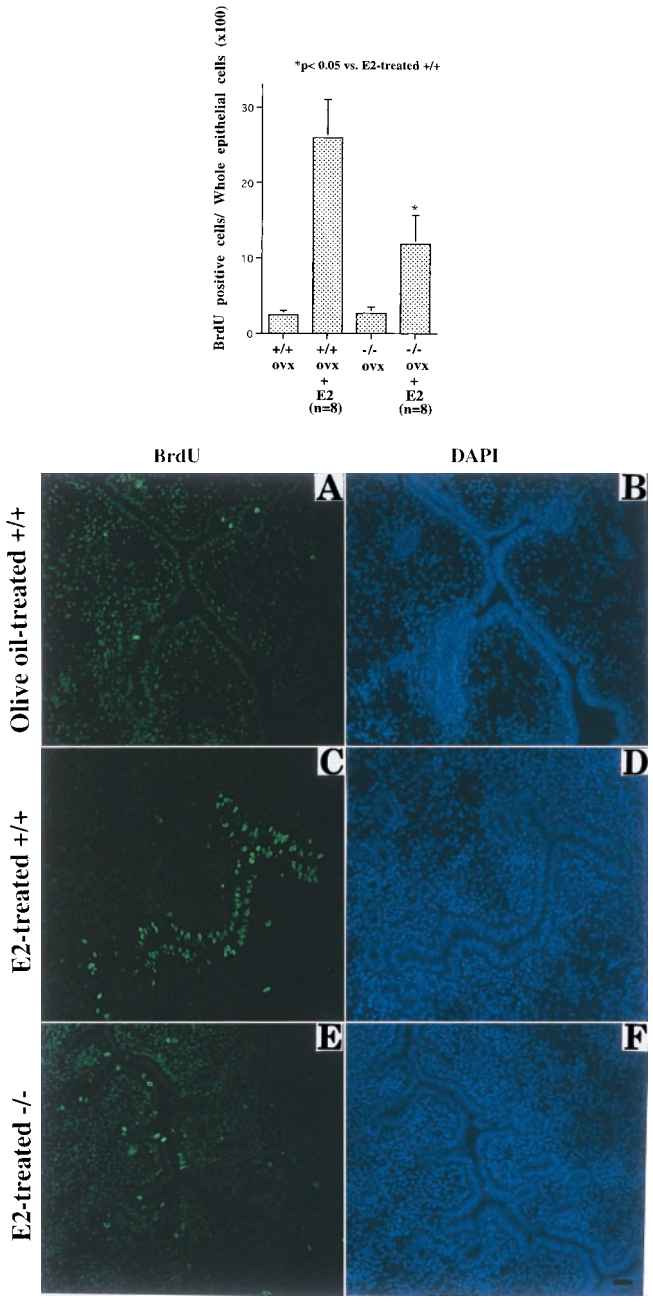

Fig. 4. Progression of cell cycle in uterine cells of F2 offspring by estrogen administration. (*Upper*) The ratio of BrdUrd-positive epithelial cells induced by estrogen in F2 offspring. Frozen sections from E2-treated wild-type mice (+/+), homozygotes (−/−), and non-E2-treated mice were immunostained by using anti-BrdUrd antibody, and then the BrdUrd labeling index was calculated. The increase in the ratio of the BrdUrd labeling index between E2-treated homozygotes (−/−) and olive oil-treated homozygotes (−/−) is 59% lower than the increase between E2-treated wild-type mice (+/+) and olive oil-treated wild-type mice (+/+). The *t* test shows a significant difference ($P < 0.05$) between E2-treated wild-type mice (+/+) ($n = 8$) and E2-treated homozygotes (−/−) ($n = 8$). (*Lower*) Immunohistochemistry of BrdUrd-positive cells in the uterus of F2 offspring. Immunoreactivity of BrdUrd-positive cells is shown in *A*, *C*, and *E*. 4′,6′ diamino-2-phenylindole staining is shown in *B*, *D*, and *F*. *A* and *B* indicate olive oil-treated wild-type mice (+/+). *C* and *D* indicated E2-treated wild-type mice (+/+), and *E* and *F* indicate E2-treated homozygotes (−/−). Note the low numbers of BrdUrd-positive epithelial cells in olive oil-treated wild-type mice (+/+) (*A*) and E2-treated homozygotes (−/−) (*E*), as compared with that in E2-treated wild-type mice (+/+) (*C*). (Bar = 125 μm.)

We previously isolated the efp gene with palindromic ERE as one of the direct downstream targets of ERα (23). Interestingly, efp mRNA increased within 2 h in the uterus in response to estrogen. Its coexistence with ERα and the kinetics of uterine cell growth raised the possibility that efp might be one of the mediators of estrogen action related to uterine development (24).

This time, we have generated efp knockout mice to examine this hypothesis. They showed underdevelopment of the uterus as a major phenotype. In addition, much decreased response to estrogen also was demonstrated by the smaller increase of uterine wet as well as dry weight and also by a lower cell cycle progression from Go/G1 to S phase in homozygotes than in wild-type mice, as shown by the lower incorporation of BrdUrd into uterine cell DNA. Serum 17β-estradiol and gonadotropin levels in homozygotes, however, were within normal range, and the ERα protein also was detected certainly in the uterus of homozygotes (data not shown). These findings suggest that efp would be required for the full biological response to estrogen in the uterus.

We have herein put forward evidence that efp might promote the uterine cells to enter the S phase and proliferate, but its molecular mechanisms remain to be elucidated. It is reported that protooncogenes, growth factors, and cyclins such as c-fos (43, 44), c-myc (43), c-jun (45), insulin-like growth factor 1 (46), transforming growth factor α (47), vascular endothelial growth factor (48), heparin-binding–epidermal growth factor (49), and cyclin D1 (49, 50) might be direct mediators of uterine cell proliferation. They are up-regulated within 0.5–2 h for protooncogenes, 2–6 h for growth factors, and 8–12 h for cyclin D1 in the uterus by estrogen. Earlier response (within 2 h) of efp to estrogen than these growth factors and cyclins in the uterus leads us to consider that efp might be present upstream of them, although, in preliminary studies, no significant immunohistochemical differences were found in expression of these putative mediators between wild-type and efp homozygotes (data not shown). Their direct responsiveness, but not the secondary one, to estrogen described previously (43–49) might suggest a possible role of efp via interaction with these cell cycle regulators. It would therefore be interesting to clarify how efp interacts with some cdk-cyclin complexes during progression through Go/G1 phase in uterine cells because it is also reported that estrogen induces the activation of cdk4-cyclin D1 and/or cdk2-cyclin E complexes during G1-S phase in MCF-7 human mammary carcinoma cells (51–54) and in the uterus (50).

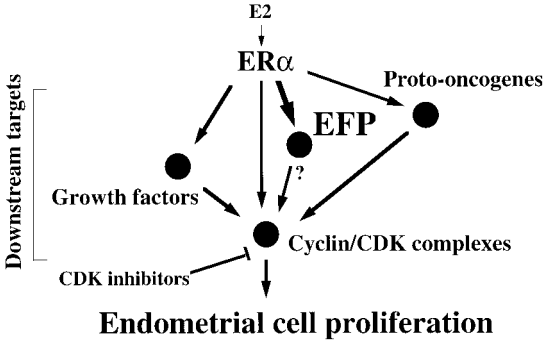

Fig. 5. Downstream signaling under ERα involved in endometrial cell proliferation. A schematic view of a downstream cascade under ERα involved in endometrial cell proliferation is shown. efp is one candidate of downstream targets under ERα, and the endometrial cell proliferation induced by 17β-estradiol (E2) was partially inhibited in the mice which lacked the efp gene whereas it is known that several protooncogenes, growth factors, and cyclins up-regulated by estrogen in the uterus might be positive mediators of uterine cell proliferation (see *Discussion*). This work demonstrates that efp also might be one of positive mediators involved in cell proliferation in the uterus.

A quite poor response of interstitial edema induced by estrogen in the uterus of efp homozygotes also was demonstrated in the study. Although previous studies (48, 55) have reported that the increase of the fluid uptake by the uterus, probably attributable to the enhanced microvascular permeability after estrogen treatment, is at least partially mediated by growth factors such as vascular endothelial growth factor, insulin-like growth factor, and epidermal growth factor, there were no significant differences in the amounts of these factors in interstitial cells of efp homozygotes when immunohistochemically compared with wild type mice (data not shown). Further studies are needed to clarify the relationships between the growth factors and efp that might also mediate water imbibition.

This work demonstrates that a direct estrogen-responsive gene, efp, actually mediates one of the estrogen actions: i.e., epithelial growth in a target organ, the uterus, under the circumstance in which a variety of genes up-regulated by estrogen make direct controls obscure and difficult to determine (Fig. 5). However, relatively mild infantile uterine development in efp homozygotes as compared with the severe hypoplastic uterus shown in ERα null-mutant mice leads us to suppose the presence of some compensation mechanisms by other downstream targets and/or different pathways operating for uterine development. At any rate, it may be concluded that efp is required at least for maximal uterine cell growth to develop a normal size uterus and for full response to estrogen. Moreover, this study also supports the notion that the activation of downstream targets via ERE known as the classical cascade is functioning as the significant pathway for maintenance of the uterus. Furthermore, the results in the present mouse model might suggest a possible role of efp involved in dysmenorrhea with hypoplastic uterus in human and/or in the pathogenesis of estrogen-dependent uterine proliferative disease such as endometriosis and endometrial atypical hyperplasia.

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