ESTROGENS regulate normal development of estrogen-responsive tissues such as the mammary glands and uterus via proliferative action and stimulate pathogenesis of estrogen-responsive tumors in these tissues [1]. The current strategy of endocrine therapy for breast and endometrial cancers involves blocking the proliferative action of estrogens using estrogen antagonists and aromatase inhibitors [2]. There is, however, accumulating evidence for anti-proliferative and anti-tumor actions of estrogens in a variety of cells in vivo and in vitro. Estrogens inhibit proliferation of normal cells such as vascular smooth muscle cells [3] and even of uterine cells in primary culture [4]. Estrogens inhibit carcinogen-induced mammary cancer [5] and growth of colon cancer [6]. Interestingly, the anti-proliferative action of estrogens is observed in estrogen receptor α (ERα)-negative cells that are manipulated to express exogenous ERα [7]. Pituitary lactotrophs are also well known estrogen-responsive cells. Lactotrophs are unique in that both proliferative and anti-proliferative actions of estrogens are found within the same lactotrophs in a cell context-dependent manner: 17β-estradiol (E2) stimulates lactotroph proliferation in the presence of serum while it inhibits proliferation in the presence of growth factors such as insulin-like growth factor-1 (IGF-1) [8, 9]. Research regarding the anti-proliferative action of estrogens may provide new insights into the mechanism of estrogen regulation of cell proliferation and is expected to contribute to the development of novel therapeutic approaches to estrogen-responsive tumors.

The developmental and physiological actions of estrogens are mostly mediated by the nuclear ERα and ERβ, which regulate expression of target genes by acting as ligand-activated transcription factors [10]. The nuclear ER directly binds to specific DNA sequences
known as estrogen-response elements (EREs) in the regulatory regions of target genes and activates transcription through the recruitment of coactivator proteins. Alternatively, the nuclear ER can bind to other transcription factors such as AP-1 and Sp1 and indirectly modulate gene expression through protein-protein interactions [11, 12]. In contrast to these rather slow genomic actions mediated by nuclear ERs, estrogens also induce a number of rapid but transient effects on second messenger levels and protein kinase signaling [13]. Rapid effects of estrogens that involve non-genomic mechanisms seem to be independent of transcriptional activation mediated by nuclear ERs, and instead appear to be mediated by receptors located in the cytoplasmic membrane. There have been several reports identifying putative membrane ERs, which are distinct from the nuclear ERα and ERβ. One of these is G protein-coupled estrogen receptor 1 (Gper1), which upon binding to estrogens stimulates adenylyl cyclase and EGFR transactivation leading to Ca2+ mobilization and activation of the mitogen-activated protein kinase Erk1/2 and PI3K/Akt signaling pathways [14, 15]. However, it is still controversial that Gper1 is a membrane ER that mediates the non-genomic, rapid effects of estrogens because Pedram et al. [16] have shown that E2 fails to activate the rapid multiple pathways in cells that lack classical ERα and ERβ even when Gper1 is present.

Several groups report conflicting results regarding the cellular localization of Gper1 in the cytoplasmic membrane or the intracellular endoplasmic reticulum [17, 18]. A high-affinity, limited capacity single binding site-specific [3H]E2 has been detected in cytoplasmic membranes of several cell types that express Gper1 but lack nuclear ERs [19, 20]. Immunohistochemical and fluorescence-activated cell sorting studies showed that the localization of endogenous Gper1 and tagged Gper1 was observed in the cytoplasmic membrane of uterine epithelial cells [21], hippocampal cells [22], and HEK-293 cells [20]. These findings suggest Gper1 is located in the cytoplasmic membrane. In contrast, green fluorescence protein-labeled Gper1 was detected in the endoplasmic reticulum of COS7 cells as revealed by confocal fluorescence microscopy, and the intracellular expression of the labeled-Gper1 and endogenous Gper1 was confirmed by immunostaining with anti-Gper1 [23, 24].

We have previously shown that suppression of Bcl3 gene expression is involved in the anti-proliferative action of estrogens in pituitary lactotrophs [25]. However, these results do not necessarily exclude the possibility that estrogens bind to membrane ERs to induce anti-proliferative action. Therefore, we investigated whether the anti-proliferative action of estrogens on pituitary lactotrophs is mediated by the classical nuclear ER pathway or by the recently postulated ER membrane pathway. Furthermore, we determined whether Gper1 is involved in the regulation of lactotroph proliferation.

**Materials and Methods**

**Reagents**

E2, IGF-1, diethylstilbestrol (DES), BSA-conjugated E2 (β-estradiol 6-(O-carboxymethyl)oxime: BSA) (BSA-E2), and 5-bromo-2′-deoxyuridine (BrdU) were purchased from Sigma (St. Louis, MO). G-1 and G-15 were purchased from Cayman (Ann Arbor, MI) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. BSA-E2 purification was carried out as described elsewhere [26]. To prepare dextran-coated charcoal-treated horse serum (DCC-HS), horse serum was treated with activated charcoal [27]. E2, DES, BSA-E2, G-1 and G-15 were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in culture medium was 0.05%.

**Cell culture**

Experiments were approved by the Ethical Committee for Animal Experiments of the University of Yamanashi. Seven-week-old female Wistar rats, purchased from Japan SLC (Shizuoka, Japan), were used to obtain anterior pituitary cells for primary culture. Anterior pituitary cells were dispersed as described previously [25]. A 100 µL aliquot of a cell suspension containing 2.0×10^5 pituitary cells was plated on poly-D-lysine-coated 35 mm culture dishes for proliferation studies and promoter activity assays, and a 300 µL aliquot containing 6.0×10^5 pituitary cells was used for quantitative real-time PCR (qRT-PCR) and immunoblotting analysis. For the promoter activity assays, cell suspensions were mixed with adenovirus vectors at appropriate multiplicities of infection (MOIs) when the cells were plated. The cells were allowed to attach to the surface of the dishes and pre-cultured with a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s Nutrient Mix F-12 without phenol red, containing 15 mM
HEPES, penicillin, and streptomycin (DMEM/F12) containing 500 ng/mL insulin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air for 1 day. After 1 day of pre-culture, the pituitary cells were washed with DMEM/F12 and cultures were initiated with a serum-free, chemically defined medium [8]. In proliferation studies, after 1 day of serum-free culture, the pituitary cells were treated with agents for 24 h. To label proliferating pituitary cells, the cultured cells were treated with 200 µM BrdU for 3 h prior to the end of culture. The cells were re-dispersed, attached to poly-d-lysine-coated glass slides, and fixed with ice-cold methanol for double immunostaining for prolactin (PRL) and BrdU.

**Immunostaining**

To determine the BrdU-labeling index, pituitary cells labeled with BrdU were double immunostained for BrdU and PRL as described previously [8]. Immunostained slides were examined using a fluorescence microscope equipped with a dual-band mirror unit for fluorescein isothiocyanate and Texas Red. A total of 1,000 PRL-immunoreactive cells were examined in randomly chosen fields for each slide to determine the BrdU-labeling index, which was the percentage of cells immunoreactive for both PRL and BrdU of the total PRL-immunoreactive cells counted.

**Promoter activity assays using recombinant adenovirus vectors**

To measure ERE-mediated promoter activity, reporter genes were introduced into cells with the aid of adenovirus vectors as described previously [27]. Pituitary cells were infected with Ad-ERE.TK/Luc and Ad-TK/rLuc adenovirus vectors, both at 2 MOIs, when the cells were plated. Cells were harvested with 200 µL of Passive Lysis Buffer (Promega, Madison, WI) according to the manufacturer’s protocol. The cell lysates were centrifuged to remove cell debris and subjected to reporter assays using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Light intensity was measured with a luminometer (BLR-201; Aloka, Tokyo, Japan). Promoter activity levels measured by Ad-ERE.TK/Luc were normalized to those measured by Ad-TK/rLuc.

**Quantitative real-time PCR (qRT-PCR)**

RNA isolation and cDNA synthesis were performed as described previously [25]. Reverse transcriptase reactions were amplified on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster, CA) using Thunderbird™ SYBR® qPCR Mix (Toyobo, Osaka, Japan). The forward and reverse primer sequences are shown in Table 1. After the initial denaturation step at 95 °C for 1 min, amplification was performed for 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Thermal amplification was followed by dissociation-curve analysis to confirm the specificity of the amplification products. The mRNA levels were calculated by the 2-ΔΔCₜ method [28] using acid ribosomal phosphoprotein P0 (Arbp) as an endogenous reference.

**Immunoblotting**

Immunoblotting was performed as described previously [29]. Pituitary cells were lysed with ice-cold lysis buffer composed of 20 mM Tris, pH 7.6, 137 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and a protease inhibitor cocktail (aprotinin, bestatin, leupeptin, and pepstatin A) (Santa Cruz Biotechnology, Santa Cruz, CA), and a whole-cell extract of soluble proteins was obtained by centrifugation at 17,500 g for 15 min at 4 °C. Supernatant aliquots containing equal amounts of total protein (5-20 µg) from each sample were denatured in NuPAGE® LDS Sample Buffer and NuPAGE® Reducing Agent (Invitrogen, Carlsbad, CA) for 10 min at 70 °C and separated on a NuPAGE® 10% Bis-Tris gel (Invitrogen) under denaturing conditions in NuPAGE® MOPS SDS Running Buffer (Invitrogen), and then transferred to polyvinylidene difluoride membranes with the iBlot® Dry Blotting System (Invitrogen) according to the manufacturer’s protocol. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and then incubated with primary antibodies in TBST containing 5% BSA at 4 °C overnight. The primary antibodies used were as follows: anti-phosphorylated p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:2,000 dilution), anti-Akt (1:2,000 dilution), and anti-phosphorylated Akt (Ser473) (1:2,000 dilution) were purchased from Cell Signaling Technology (Beverly, MA), anti-Erk1 (1:4,000 dilution) from Becton Dickinson Biosciences (Bedford, MA), and anti-β-actin (1:10,000 dilution) from Sigma. The primary antibodies were immunoreacted with a horseradish peroxidase-conjugated anti-rabbit or mouse IgG.
antibody (Amersham Biosciences; 1:10,000 dilution in TBST). Immunoreactive proteins were detected using the ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, England) according to the manufacturer’s instructions, and the signal was detected with a luminoimage analyzer (LAS-4000; Fuji Photo Film, Tokyo, Japan). Protein levels were assessed by densitometric analysis using ImageQuant TL software (GE Healthcare).

Statistical analysis
Experiments were carried out using three culture dishes per experimental group and replicated at least three times with separate batches of cell preparations unless otherwise stated. The results are expressed as the means ± the standard errors of the mean (SEM). Differences between groups were statistically analyzed using one-way ANOVA followed by Fisher’s protected least significant difference test.

Results

The anti-proliferative action of E2 on lactotrophs is not mediated by membrane ERs.
To investigate whether the inhibitory action of E2 on IGF-1-induced lactotroph proliferation is mediated by membrane ERs, we examined the effects of cytoplasmic membrane-impermeable BSA-conjugated E2, which is known to activate cytoplasmic membrane ERs but not nuclear ERs [30, 31], on lactotroph proliferation. As shown previously [8], treatment with 30 ng/mL IGF-1 alone markedly stimulated basal proliferation of lactotrophs in primary culture as determined by BrdU labeling, which was significantly suppressed to 38% by 1 nM E2 ($p<0.05$) (Fig. 1A). BSA-E2 at 1 nM, a concentration that did not suppress lactotroph proliferation, had no effect on

| Table 1 Primers used for qRT-PCR analysis |
|-------------------------------------------|
| Gene | Forward primer (5′→3′) | Reverse primer (5′→3′) |
|------|-------------------------|------------------------|
| Arhp | GCCTCACCTCACATCAAAATGG | TTTTCAACTGGAAGAAGGT |
| Arhgap18 | GGTTGCCCAGGCTACCTAA | GCAACGTCCCAATATTCTC |
| Baf3 | GAGGTGGAGCAGAGGAAATGCT | GGAGGGCTACAGTCAAGTG |
| Bcl3 | GTCCCTCCGAGAGGTGCTT | AGCGAGGTGAGGTGAGGT |
| c-fos | AGACATAGGAGAGGGCCAAA | AGCGAGGTGAGGTGAGGT |
| c-fos | CCGCGGATGAGGCACTTACCT | ACTCGAGGTGAGGTGAGGT |
| c-myc | ATGCAGGCTCAAAAGGCTTT | GCCGGGATGAGGTGAGGT |
| Ccn2a | CCTGCAACACTGAAAAGTGA | GGAGGGCTACAGTCAAGTG |
| Cen3d | AGTCGCTCCGCAATGTGGAATG | AAGGGGCTACAGTCAAGTG |
| Cen3d | GTACTCTCCAGTGGCAAAAGC | TCTGAGGAGATGAGGTGAGGT |
| Dicer2 | CTGGCCTCATGGTCTCTGTTG | CCGGAGGCTACAGTCAAGTG |
| Fox1l | CCAAAAGCAGCTCAGTGTTT | GCTTACTCTGCAAGAGGAG |
| G6od | TGAAGAAGAATCTGAGGACAAACC | TCTGAGGAGATGAGGTGAGGT |
| Igf1 | CCCTCCCTCACTACAGAATCTAAT | GGAGGGCTACAGTCAAGTG |
| Mek1 | GAAACTGAGATCTCAACAGAATCTAAT | GGAGGGCTACAGTCAAGTG |
| Nfat1 | CCGCAAAATCTTACCCGACACT | GAGGGGCTACAGTCAAGTG |
| Palmd | CCCGGCTGAAAAGAAGCAGG | CAGAGGTGAGGTGAGGT |
| Pena | TGGCACTCTCACAGCTGCTG | GAGGGGCTACAGTCAAGTG |
| Fdlim3 | GTGATGGGAGATGAGAGGAG | GAGGGGCTACAGTCAAGTG |
| Pim3 | GACAGGACTCTCAAGCCTTAAAT | CACTGAGGCTACAGTCAAGTG |
| Prkag2 | GTTTGCTGGATAGGAAGGTCTAG | GAGGGGCTACAGTCAAGTG |
| Rasd1 | CCAGGACTCTACAGGCAATAC | GAGGGGCTACAGTCAAGTG |
| Sta1 | ATGCTGCTCCAGTGGCAAAAGC | GAGGGGCTACAGTCAAGTG |
| Tcfcp2l1 | CAGCGCTGCTGAGATGAGGAG | GAGGGGCTACAGTCAAGTG |
| Wnt4 | CTGCAAGCTCTACTGCTTGTG | GAGGGGCTACAGTCAAGTG |
Fig. 1 Effects of BSA-E2 on proliferation of pituitary lactotrophs, ERE-mediated promoter activity, and mRNA expression of estrogen-responsive genes

(A), Pituitary cells in primary culture were treated with 30 ng/mL IGF-1 alone or in combination with 1 nM E2, or 1 or 100 nM BSA-E2 for 24 h. Pituitary cells were labeled with BrdU for the last 3 h of culture and subjected to PRL/BrdU immunostaining. (B), Pituitary cells co-infected with Ad-ERE.TK/Luc and Ad-TK/rLuc, each at 2 MOI, were treated with 30 ng/mL IGF-1 alone or in combination with 1 nM E2, or 1, 10, or 100 nM BSA-E2 for 24 h. Cell lysates were subjected to dual luciferase assays for ERE-mediated promoter activity. (C), Pituitary cells were treated with 30 ng/mL IGF-1 alone or in combination with 1 nM E2 or 1 or 100 nM BSA-E2 for 4 h. mRNA expression was determined by qRT-PCR analysis using primers listed in Table 1. The BrdU labeling index, normalized ERE promoter activity, and mRNA expression are expressed as a fold increase relative to the non-treated group. Data were means ± SEM of triplicate determinations from a representative experiment. * Significantly different from the group treated with IGF-1 alone at P<0.05.
ERE activity. Importantly, 10 and 100 nM BSA-E2 concentrations, which were effective in suppressing lactotroph proliferation, increased ERE activity in a dose-dependent manner \((p<0.05)\). We verified the results of ERE activity by analyzing mRNA expression of pituitary estrogen-responsive genes [25]. Treatment with 1 nM E2 significantly up- and down-regulated mRNA expression of a variety of genes 4 h later \((p<0.05)\) (Fig. 1C). BSA-E2 at 1 nM neither up-regulated nor down-regulated most of these genes; only mRNA expression of Wnt4 was slightly but significantly increased. In contrast, 100 nM BSA-E2 modulated mRNA expression of estrogen-responsive genes similarly to 1 nM E2. These results suggest that BSA-E2 does not inhibit IGF-1-induced lactotroph proliferation at concentrations that do not affect estrogen-responsive gene expression.

**The anti-proliferative action of E2 on lactotrophs is mediated by nuclear ERs.**

DES is known to selectively activate nuclear ERs but not membrane ERs [19, 32]. We utilized this property of DES to investigate whether the inhibitory action of E2 on IGF-1-induced lactotroph proliferation is mediated by nuclear ERs. First, we verified that the potencies of 1 nM E2 and equimolar DES were similar for modulating mRNA expression of 4 estrogen-responsive genes at 3 and 12 h after treatment (Fig. 2A). Next, we found that E2 and DES at this concentration both inhibited IGF-1-induced lactotroph proliferation to a similar degree \((p<0.05)\) (Fig. 2B). These results suggest that the anti-proliferative action of E2 on lactotroph proliferation is mediated exclusively by classical nuclear ERs.

![Fig. 2](#)

**Fig. 2** Effects of DES on the proliferation of pituitary lactotrophs and mRNA expression of estrogen-responsive genes

(A). Pituitary cells in primary culture were treated with vehicle, 1 nM E2, or 1 nM DES for 3 (left panel) and 12 h (right panel). mRNA expression was determined by qRT-PCR analysis. mRNA expression is expressed as a fold increase relative to the vehicle-treated group. Data were means ± SEM of triplicate determinations from a representative experiment. * Significantly different from the vehicle-treated group (open bar) at \(P<0.05\). (B). Pituitary cells in primary culture were treated with 30 ng/mL IGF-1 alone or in combination with 1 nM E2 or BSA-E2 for 24 h. The pituitary cells were labeled with BrdU for the last 3 h of culture and subjected to PRL/BrdU immunostaining. The BrdU labeling index is expressed as a fold increase relative to the non-treated group (open bar). * Significantly different from the group treated with IGF-1 alone (solid bar) at \(P<0.05\).
G-1 mimics the anti-proliferative action of E2 on lactotrophs.

Our finding that classical nuclear ERs mediate the anti-proliferative action of E2 on lactotroph proliferation does not exclude the possibility that the membrane ER Gper1 is also involved in the anti-proliferative action of E2, since the subcellular localization of Gper1 in either the cytoplasmic membrane or endoplasmic reticulum remains undetermined. Therefore, we used G-1, a Gper1 agonist [33], to test whether Gper1 also inhibits lactotroph proliferation. Similar to E2, G-1 at concentrations ranging from 10 nM–1 µM significantly inhibited IGF-1-induced lactotroph proliferation with a maximal inhibition of 39% (p<0.05) (Fig. 3 left panel). These results indicate that activation of Gper1, which may be located on the endoplasmic reticulum membrane, is capable of inhibiting IGF-1-induced lactotroph proliferation to a degree similar to E2.

Although E2 shows anti-proliferative action in the presence of IGF-1, estrogens also have proliferative effects on lactotrophs in the presence of serum [8]. Therefore, we next sought to determine whether G-1 also mimics the proliferative action of estrogens. Treatment with 10% DCC-HS alone did not change basal proliferation (Fig. 3 right panel). E2 in combination with DCC-HS significantly stimulated lactotroph proliferation to 2.2 fold (p<0.05). However, G-1 at 1 µM, a maximally effective dose for inhibiting IGF-1-induced lactotroph proliferation as determined above, had no stimulatory effect on lactotroph proliferation in the presence of DCC-HS. These results suggest that Gper1 selectively mimics the anti-proliferative action, but not the proliferative action, of E2 on lactotrophs.

To verify the non-genomic action of G-1 on lactotroph proliferation, we compared the effects of E2 and G-1 on mRNA expression of known estrogen-responsive genes by qRT-PCR analysis. We selected 17 estrogen-responsive genes that have previously been shown to be up- or down-regulated in pituitary cells after treatment with E2 in combination with IGF-1 by DNA microarray analysis [25]. E2 markedly up-regulated mRNA expression of genes such as Batf3, c-myc, Dhcr24, Rasd1, Tcfcp211, and Wnt4 after both 4 and 24 h (Table 2). E2 consistently down-regulated gene expression of c-jun, Cnd3, Giot1, Igf1, Mybl1, Nptx1, Pcna, and Stc1 at both time points. However, treatment with 1 µM G-1 either did not modulate mRNA expression of estrogen-responsive genes at either time point or, for a few genes such as Fosl1, Igf1, and Stc1, modestly inhibited mRNA expression at one time point.

![Fig. 3 Effects of G-1 on the proliferation of pituitary lactotrophs](image-url)

Pituitary cells in primary culture were treated with 30 ng/mL IGF-1 alone or in combination with 1 nM E2 or 10, 100 nM, or 1 µM G-1 (left panel) and 10% DCC-HS alone or in combination with 1 nM E2 or 1 µM G-1 for 24 h (right panel). The pituitary cells were labeled with 200 µM BrdU for the last 3 h of culture and subjected to PRL/BrdU immunostaining. The BrdU labeling index is expressed as a fold increase relative to the non-treated group (open bars). Data were means ± SEM of triplicate determinations from a representative experiment. * Significantly different from the groups treated with IGF-1 and DCC-HS alone (solid bars) in left and right panel, respectively, at P<0.05.
Table 2 Effects of E2 and G-1 on mRNA expression of estrogen-responsive genes in pituitary cells

| Gene                                         | Symbol      | Fold change (mean ± SEM) |
|----------------------------------------------|-------------|--------------------------|
|                                              |             | 4 h          | 24 h          |
|                                              |             | IGF-1       | IGF-1+E2      | IGF-1+G-1 | IGF-1       | IGF-1+E2      | IGF-1+G-1 |
| Basic leucine zipper transcription factor, ATF-like 3 | Batf3       | 1.36 ± 0.11 | 7.56 ± 0.31* | 1.77 ± 0.28 | 1.38 ± 0.10 | 5.51 ± 0.08* | 1.24 ± 0.07 |
| FBJ osteosarcoma oncogene                     | c-fos       | 0.82 ± 0.15 | 0.90 ± 0.06 | 0.75 ± 0.11 | 0.74 ± 0.01 | 1.54 ± 0.06* | 0.67 ± 0.11 |
| Jun proto-oncogene                             | c-jun       | 1.14 ± 0.10 | 0.83 ± 0.02* | 1.03 ± 0.04 | 1.08 ± 0.08 | 0.62 ± 0.02* | 0.90 ± 0.05 |
| Myelocytomatosis oncogene                      | c-myc       | 1.95 ± 0.08 | 3.53 ± 0.07* | 1.92 ± 0.03 | 1.33 ± 0.09 | 2.81 ± 0.12* | 1.18 ± 0.07 |
| Cyclin D3                                     | Ccnd3       | 0.98 ± 0.15 | 0.62 ± 0.02* | 0.91 ± 0.10 | 1.20 ± 0.03 | 0.72 ± 0.16* | 1.19 ± 0.07 |
| 24-dehydrocholesterol reductase               | Dhcr24      | 2.29 ± 0.22 | 3.86 ± 0.13* | 2.62 ± 0.09 | 3.06 ± 0.13 | 4.16 ± 0.12* | 3.04 ± 0.14 |
| Fos-like antigen 1                             | Fosl1       | 2.84 ± 0.07 | 1.20 ± 0.07* | 2.50 ± 0.15* | 1.07 ± 0.08 | 1.31 ± 0.02* | 0.94 ± 0.03 |
| Gonadotropin inducible ovarian transcription factor 1 | Giot1       | 0.57 ± 0.04 | 0.17 ± 0.01* | 0.56 ± 0.04 | 0.70 ± 0.03 | 0.49 ± 0.03* | 0.64 ± 0.02 |
| Insulin-like growth factor 1                   | Igf1        | 0.63 ± 0.05 | 0.42 ± 0.03* | 0.56 ± 0.06 | 1.03 ± 0.06 | 0.34 ± 0.04* | 0.82 ± 0.04* |
| Myeloblastosis oncogene-like 1                 | Mybl1       | 1.32 ± 0.07 | 0.53 ± 0.03* | 1.19 ± 0.03 | 3.00 ± 0.14 | 0.71 ± 0.01* | 2.91 ± 0.19 |
| Neuronal pentraxin 1                           | Nptx1       | 5.07 ± 0.33 | 0.88 ± 0.01* | 4.36 ± 0.47 | 1.06 ± 0.08 | 0.63 ± 0.06* | 0.87 ± 0.08 |
| Proliferating cell nuclear antigen             | PcnA        | 1.31 ± 0.06 | 0.89 ± 0.02* | 1.21 ± 0.09 | 2.37 ± 0.07 | 1.23 ± 0.15* | 2.35 ± 0.10 |
| Protein kinase, AMP-activated, gamma 2 non-catalytic subunit | Prkag2      | 1.42 ± 0.07 | 2.24 ± 0.06* | 1.23 ± 0.05 | 1.16 ± 0.12 | 1.40 ± 0.03 | 1.17 ± 0.03 |
| RAS, dexamethasone-induced 1                   | Rasd1       | 0.85 ± 0.02 | 2.96 ± 0.01* | 0.77 ± 0.03 | 0.69 ± 0.02 | 2.89 ± 0.10* | 0.63 ± 0.00 |
| Stanniocalcin 1                                | Stc1        | 2.70 ± 0.22 | 1.21 ± 0.07* | 2.20 ± 0.23 | 2.85 ± 0.12 | 1.28 ± 0.07* | 2.04 ± 0.14* |
| Transcription factor CP2-like 1                | Tcfcp2l1    | 0.66 ± 0.10 | 2.92 ± 0.03* | 0.68 ± 0.07 | 0.67 ± 0.03 | 2.80 ± 0.20* | 0.60 ± 0.02 |
| Wingless-related MMTV integration site 4       | Wnt4        | 1.40 ± 0.01 | 8.24 ± 0.27* | 1.31 ± 0.13 | 1.18 ± 0.03 | 6.18 ± 0.29* | 0.91 ± 0.05 |

Pituitary cells were treated with vehicle or IGF-1 alone or in combination with 1 nM E2 or 1 µM G-1 for 4 and 24 h. mRNA expression of 17 selected estrogen-responsive genes was determined by qRT-PCR analysis. Data are presented as the mean ± SEM of fold changes relative to the vehicle-treated group. * Significantly different from the IGF-1 alone-treated group at \( P<0.05 \).

G-1 does not affect IGF-1-induced activation of Akt and Erk1/2.

Growth factors, including IGF-1, stimulate cell proliferation via activation of the PI3K/Akt and Erk pathways. Therefore, it is possible that inhibition of IGF-1-induced lactotroph proliferation by G-1 is due to inhibition of IGF-1 activation of Akt and Erk. We investigated the effects of G-1 on IGF-1-induced phosphorylation of Akt and Erk1/2 by Western blotting analysis. Treatment of pituitary cells with IGF-1 elicited rapid and marked phosphorylation of Akt as early as 5 min, and this phosphorylation consistently continued up to 4 h (Fig. 4). Erk1/2 phosphorylation was found 5 min after IGF-1 treatment, but this change was modest and transient compared with Akt phosphorylation. There was no difference in Erk1/2 phosphorylation between vehicle and IGF-1 1 and 4 h later. G-1 did not change Akt phosphorylation at any time point, and did not change Erk1/2 phosphorylation at 5 min after IGF-1 treatment.
**G-15 does not affect the anti-proliferative action of E2 on lactotrophs.**

To test whether Gper1 activation mediates the anti-proliferative action of E2 on lactotrophs, we examined the effects of G-15, a Gper1 selective antagonist [34], on E2 inhibition of IGF-1-induced lactotroph proliferation. Treatment with 1 nM E2 in combination with 10 nM G-15 significantly inhibited IGF-1-induced lactotroph proliferation to a similar degree as treatment with E2 alone (*p*<0.05) (Fig. 5). Even a ten-fold higher concentration of G-15 was ineffective in altering E2 inhibition of IGF-1-induced proliferation. These results suggest that Gper1 activation does not mediate the anti-proliferative action of E2.

---

**Fig. 4** Effects of G-1 on the phosphorylation of Akt and Erk1/2 in pituitary cells

Pituitary cells were treated with vehicle (V), 30 ng/mL IGF-1 alone (I) or in combination with 1 μM G-1 (I+G), or G-1 alone (G) for 5 min, and 1 and 4 h. Cell lysates were subjected to immunoblotting analysis using antibodies against Akt and Erk1/2 proteins, their phosphorylated forms (pAKT and pErk1/2), and β-actin protein.

**Fig. 5** Effects of G-15 on the anti-proliferative action of E2 on lactotrophs

Pituitary cells in primary culture were treated with 30 ng/mL IGF-1 alone, IGF-1 and 1 nM E2, or IGF-1 with 1 nM E2 and 10 or 100 nM G-15 for 24 h. Pituitary cells were labeled with BrdU for the last 3 h of culture and subjected to PRL/BrdU immunostaining. The BrdU labeling index is expressed as a fold increase relative to the non-treated group (open bar). Data were means ± SEM of triplicate determinations from a representative experiment. * Significantly different from the group treated with IGF-1 alone (solid bar) at *P*<0.05.
**Discussion**

We investigated whether the anti-proliferative action of estrogens on pituitary lactotrophs is mediated by the classical nuclear ER pathway or by the recently postulated cytoplasmic membrane-bound ER pathway. We showed that BSA-E2 at 1 nM, an equimolar concentration at which E2 exerts anti-proliferative effects, did not inhibit IGF-1-induced lactotroph proliferation, while higher concentrations of BSA-E2 inhibited it. However, the anti-proliferative action of BSA-E2 at 100 nM was accompanied by stimulation of ERE-mediated ER transcripational activity and modulation of a variety of estrogen-responsive genes similar to 1 nM E2 in both pattern and degree. These parallel changes in inhibition of proliferation and estrogen-responsive gene expression suggest that inhibition of lactotroph proliferation by higher concentrations of BSA-E2 is not the result of stimulation of membrane ERs but is rather attributable to stimulation of nuclear ERs by appreciable amounts of free E2 within the BSA-E2 conjugation preparation. Based on the result that the potencies of 100 nM BSA-E2 for stimulating ERE-mediated ER transcriptional activity and modulating estrogen-responsive genes were similar to those of 1 nM E2, we estimate that BSA-E2 used in the present study may contain approximately 1% free E2. The greatest care must be taken in interpreting the results obtained in studies using BSA-E2 as pointed out by others [26, 35], and our studies underscore the need for verifying the exclusion of free E2 in BSA-E2 preparation.

In contrast, DES inhibited IGF-1-induced proliferation and modulated mRNA expression of estrogen-responsive genes to a similar degree as E2. Thomas et al. [19] reported that [3H]E2 bound to cytoplasmic membranes of nuclear ER-negative SKBR3 cells was displaced by antiestrogens such as tamoxifen and ICI 182,780 but not by DES. Ariazi et al. [32] showed that E2, tamoxifen, and G-1, but not DES, elicited increased cytosolic Ca$^{2+}$ in SKBR3 cells. These findings indicate that DES selectively activates nuclear ERs but not membrane ERs, in contrast to BSA-E2. Consequently, the finding that DES both inhibits IGF-1-induced lactotroph proliferation and modulates estrogen-responsive gene expression with a similar potency to E2 suggests that the anti-proliferative action of E2 is exclusively due to classical ERs localized in the nucleus or cytoplasm and not to cytoplasmic membrane-bound ERs.

The finding that classical nuclear ERs mediate the anti-proliferative action of E2 on lactotrophs does not exclude the possibility that Gper1 is also involved in the anti-proliferative action of E2 since the subcellular localization of Gper1 in either the cytoplasmic membrane or endoplasmic reticulum remains undetermined. Therefore, we tested whether the anti-proliferative action of E2 is mimicked by G-1. The Gper1 agonist G-1, which activates multiple cellular signaling pathways via Gper1 but does not activate nuclear ERα and ERβ, has yielded novel insights into the physiological roles of Gper1 [17]. We found that similar to E2, G-1 inhibited IGF-1-induced lactotroph proliferation in a dose-dependent manner, but did not modulate mRNA expression of estrogen-responsive genes. These results suggest that Gper1 activation mimics the anti-proliferative action of E2 via non-genomic action. G-1 selectively binds to Gper1 and activates it at concentrations ranging 1 nM–1 µM [33]. The effective concentrations of G-1 in our studies were comparable to these and those reported elsewhere [36–38] excluding the possible Gper1-independent action of G-1 found at µM concentrations [39, 40].

The finding of the present study that Gper1 mimics the anti-proliferative action of E2 on lactotrophs is consistent with other studies showing that Gper1 modulates cell proliferation in a variety of cells. Gper1 mediates the proliferative action of estrogens and tamoxifen in endometrial [41, 42], thyroid [43], and ovarian cancer cells [36], and Gper1 activation stimulates cell proliferation in ERα-negative breast cancer cells [36]. In contrast, Gper1 activation has been shown to inhibit cell proliferation in ERα-positive breast cancer [32, 44], urothelial [37], and endothelial cells [38]. These findings suggest a dual role for Gper1 in the regulation of cell proliferation depending on cell type. It remains to be determined how Gper1 activation inhibits IGF-1-induced lactotroph proliferation. Because G-1 does not mimic the proliferative action of E2 as demonstrated in the presence of serum, it seems possible that Gper1 activation selectively affects the IGF-1 signaling pathway. Because activation of Gper1 affects phosphorylation of Akt [45, 46] and Erk [14, 21, 47] in a variety of cells, we investigated the effects of G-1 on IGF-1-induced phosphorylation of Akt and Erk1/2 in pituitary cells. We found no effect of G-1 on sustained or transitory phosphorylation of Akt and Erk1/2, respectively, suggesting that Gper1 activation acts downstream, rather than
upstream, of phosphorylation of Akt or Erk1/2 in the pathway of IGF-1-induced lactotroph proliferation.

The finding that G-1 treatment mimics the inhibitory action of E2 on IGF-1-induced lactotroph proliferation raised the question of whether Gper1 activation mediates the anti-proliferative action of E2 on lactotrophs. However, the Gper1 antagonist G-15 [34] failed to affect E2 inhibition of IGF-1-induced proliferation, suggesting that Gper1 activation mimics but does not mediate the anti-proliferative action of E2. There are several possible explanations for this. First, the anti-proliferative effect of Gper1 activation by E2 may be much less potent than that of nuclear ER activation by E2 and is overwhelmed by it. Second, nuclear ER activation may block or attenuate the signaling pathway following E2 binding to Gper1 via an interaction. Indeed, it is known that E2 treatment decreases Gper1 expression [21, 32]. A third possibility is that Gper1 is not activated by E2 and is still an orphan receptor, as suggested by Otto et al. [24].

In conclusion, we have demonstrated that E2 inhibition of lactotroph proliferation is due to nuclear ER-mediated genomic action. Our results suggest that the activation of Gper1 mimics, but does not mediate, the anti-proliferative action of E2 on lactotrophs.

Acknowledgements

The authors would like to thank Dr. A.F. Parlow and the National Hormone & Peptide Program for providing PRL antiserum. This was supported in part by the Ministry of Education, Science and Culture of Japan (Grant-in-Aid for Scientific Research 24590297, 16K08522).

Disclosure

None of the authors have any potential conflicts on interest associated with this research.

References

1. Deroo BJ, Korach KS (2006) Estrogen receptors and human disease. J Clin Invest 116: 561-570.
2. Jordan VC, O’Malley BW (2007) Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25: 5815-5824.
3. Sullivan TR Jr, Karas RH, Aronovitz M, Faller GT, Ziar JP, et al. (1995) Estrogen inhibits the response-to-injury in a mouse carotid artery model. J Clin Invest 96: 2482-2488.
4. Uchima FDA, Edery M, Iguchi T, Bern HA (1991) Growth of mouse endometrial luminal epithelial cells in vitro: functional integrity of the oestrogen receptor system and failure of oestrogen to induce proliferation. J Endocrinol 128: 115-120.
5. Huggins C, Yang NC (1962) Induction and extinction of mammary cancer. A striking effect of hydrocarbons permits analysis of mechanisms of causes and cure of breast cancer. Science 137: 257-262.
6. Kennelly R, Kavanagh DO, Hogan AM, Winter DC (2008) Oestrogen and the colon: potential mechanisms for cancer prevention. Lancet Oncol 9: 385-391.
7. Levenson AS, Jordan VC (1994) Transfection of human estrogen receptor (ER) cDNA into ER-negative mammalian cell lines. J Steroid Biochem Mol Biol 51: 229-239.
8. Kawashima K, Yamakawa K, Takahashi W, Takizawa S, Yin P, et al. (2002) The estrogen-occupied estrogen receptor functions as a negative regulator to inhibit cell proliferation induced by insulin/IGF-1: a cell context-specific antimitogenic action of estradiol on rat lactotrophs in culture. Endocrinology 143: 2750-2758.
9. Ishida M, Takahashi W, Itoh S, Shimodaira S, Maeda S, et al. (2007) Estrogen actions on lactotroph proliferation are independent of a paracrine interaction with other pituitary cell types: a study using lactotroph-enriched cells. Endocrinology 148: 3131-3139.
10. Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, et al. (2001) Mechanisms of estrogen action. Physiol Rev 81: 1535-1565.
11. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, et al. (2000) Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74: 311-317.
12. Safe S (2001) Transcriptional activation of genes by 17β-estradiol through estrogen receptor-Sp1 interactions. Vitam Horm 62: 231-252.
13. Levin ER (2009) Plasma membrane estrogen receptors. Trends Endocrinol Metab 20: 477-482.
14. Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. Mol Endocrinol 14: 1649-1660.
15. Filardo EJ, Quinn JA, Frackelton AR Jr, Bland KI (2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenyl cyclase and cAMP-
mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol 16: 70-84.

16. Pedram A, Razandi M, Levin ER (2006) Nature of functional estrogen receptors at the plasma membrane. Mol Endocrinol 20: 1996-2009.

17. Prossnitz ER, Barton M (2014) Estrogen biology: new insights into GPER function and clinical opportunities. Mol Cell Endocrinol 389: 71-83.

18. Filardo EJ, Thomas P (2012) Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. Endocrinology 153: 2953-2962.

19. Thomas P, Pang Y, Filardo EJ, Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 146: 624-632.

20. Filardo E, Quinn J, Pang Y, Graebner C, Shaw S, et al. (2007) Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology 148: 3236-3245.

21. Gao F, Ma X, Ostmann AB, Das SK (2011) GPR30 activation opposes estrogen-dependent uterine growth via inhibition of stromal ERK1/2 and estrogen receptor α (ERα) phosphorylation signals. Endocrinology 152: 1434-1447.

22. Funakoshi T, Yanai A, Shinoda K, Kawano MM, Mizukami Y (2006) G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. Biochem Biophys Res Commun 346: 904-910.

23. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307: 1625-1630.

24. Otto C, Rohde-Schulz B, Schwarz G, Fuchs I, Klewer M, et al. (2008) G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. Endocrinology 149: 4846-4856.

25. Mitsui T, Ishida M, Izawa M, Kagami Y, Arita J (2011) Inhibition of Bcl3 gene expression mediates the anti-proliferative action of estrogen in pituitary lactotrophs in primary culture. Mol Cell Endocrinol 345: 68-78.

26. Stevis PE, Deecher DC, Suhadolnik L, Mallis LM, Frail DE (1999) Differential effects of estradiol and estradiol-BSA conjugates. Mol Cell Endocrinol 140: 5455-5458.

27. Ishida M, Mitsui T, Izawa M, Arita J (2010) Absence of ligand-independent transcriptional activation of the estrogen receptor via the estrogen response element in pituitary lactotrophs in primary culture. J Steroid Biochem Mol Biol 118: 93-101.

28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT Method. Methods 25: 402-408.

29. Wang Z, Mitsui T, Ishida M, Arita J (2008) Adenovirus vectors differentially modulate proliferation of pituitary lactotrophs in primary culture in a mitogen and infection time-dependent manner. J Endocrinol 198: 209-217.

30. Vasudevan N, Kow LM, Pfaff DW (2001) Early membrane estrogenic effects required for full expression of slower genomic actions in a nerve cell line. Proc Natl Acad Sci USA 98: 12267-12271.

31. Taguchi Y, Koslowski M, Bodenner DL (2004) Binding of estrogen receptor with estrogen conjugated to bovine serum albumin (BSA). Nucl Recept 2: 5.

32. Ariazi EA, Braitou E, Yerrum S, Shupp HA, Slifker MJ, et al. (2010) The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. Cancer Res 70: 1184-1194.

33. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, et al. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. Nat Chem Biol 2: 207-212.

34. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, et al. (2009) In vivo effects of a GPR30 antagonist. Nat Chem Biol 5: 421-427.

35. Temple JL, Wray S (2005) Bovine serum albumin-estrogen compounds differentially alter gonadotropin-releasing hormone-1 neuronal activity. Endocrinology 146: 558-563.

36. Albanito L, Madeo A, Lappano R, Vivaçqua A, Rago V, et al. (2007) G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17β-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. Cancer Res 67: 1859-1866.

37. Teng J, Wang ZY, Prossnitz ER, Bjorling DE (2008) The G protein-coupled receptor GPR30 inhibits human urothelial cell proliferation. Endocrinology 149: 4024-4034.

38. Holm A, Baidetorp B, Olde B, Leeb-Lundberg LM, Nilsson BO (2011) The GPER1 agonist G-1 attenuates endothelial cell proliferation by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle. J Vasc Res 48: 327-335.

39. Holm A, Grände PO, Ludueña RF, Olde B, Prasad V, et al. (2012) The G protein-coupled oestrogen receptor 1 agonist G-1 disrupts endothelial cell microtubule structure in a receptor-independent manner. Mol Cell Biochem 366: 239-249.

40. Wang C, Lv X, Jiang C, Davis JS (2012) The putative G-protein coupled estrogen receptor agonist G-1 suppresses proliferation of ovarian and breast cancer cells in a GPER-independent manner. Am J Transl Res 4: 390-402.

41. Vivaçqua A, Bonofilio D, Recchia AG, Musti AM, Picard D, et al. (2006) The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17β-estradiol and hydroxytamoxifen in endometrial cancer cells. Mol Endocrinol 20: 631-646.

42. Lin BC, Suzawa M, Blind RD, Tobias SC, Bulun SE, et al. (2009) Stimulating the GPR30 estrogen receptor with a novel tamoxifen analogue activates SF-1 and
promotes endometrial cell proliferation. Cancer Res 69: 5415-5423.

43. Vivacqua A, Bonofiglio D, Albanito L, Madeo A, Rago V, et al. (2006) 17β-Estradiol, genistein, and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the G protein-coupled receptor GPR30. Mol Pharmacol 70: 1414-1423.

44. Ahola TM, Manninen T, Alkio N, Ylikomi T (2002) G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells. Endocrinology 143: 3376-3384.

45. Deschamps AM, Murphy E (2009) Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats. Am J Physiol Heart Circ Physiol 297: H1806-H1813.

46. Tang H, Zhang Q, Yang L, Dong Y, Khan M, et al. (2014) GPR30 mediates estrogen rapid signaling and neuroprotection. Mol Cell Endocrinol 387: 52-58.

47. Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, et al. (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17β-estradiol and phytoestrogens in breast cancer cells. J Biol Chem 279: 27008-27016.