Engineering of a Mouse for the in Vivo Profiling of Estrogen Receptor Activity
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In addition to their well known control of reproductive functions, estrogens modulate important physiological processes. The identification of compounds with tissue-selective activity will lead to new drugs mimicking the beneficial effects of estrogen on the prevention of osteoporosis and cardiovascular or neurodegenerative diseases, while avoiding its detrimental proliferative effects. As an innovative model for the in vivo identification of new selective estrogen receptor modulators (SERMs), we engineered a mouse genome to express a luciferase reporter gene ubiquitously. The constructs for transgenesis consist of the reporter gene driven by a dimerized estrogen-responsive element (ERE) and a minimal promoter. Insulator sequences, either matrix attachment region (MAR) or \( \beta \)-globin hypersensitive site 4 (HS4), flank the construct to achieve a generalized, hormone-responsive luciferase expression. In the mouse we generated, the reporter expression is detectable in all 26 tissues examined, but is induced by 17\( \beta \)-estradiol (E\(_2\)) only in 15 of them, all expressing estrogen receptors (ERs). Immunohistochemical studies show that in the mouse uterus, luciferase and ERs colocalize. In primary cultures of bone marrow cells explanted from the transgenic mice and in vivo, luciferase activity accumulates with increasing E\(_2\) concentration. E\(_2\) activity is blocked by the ER full antagonist ICI 182,780. Tamoxifen shows partial agonist activity in liver and bone when administered to the animals. In the mouse system here illustrated, by biochemical, immunohistochemical, and pharmacological criteria, luciferase content reflects ER transcriptional activity and thus represents a novel system for the study of ER dynamics during physiological fluctuations of estrogen and for the identification of SERMs or endocrine disruptors. (Molecular Endocrinology 15: 1104–1113, 2001)

INTRODUCTION

In the last decade, the use of molecular tools provided an insight on the previously unsought number of physiological functions of estrogens in mammals and on the complexity of their actions in target cells. In addition to having an impact on the knowledge of the intracellular receptor mechanisms, these findings led to a revision of the use of estrogens as therapeutic agents (1). At the present time, molecules active through estrogen receptors (ERs) are used in fertility control, endocrine dysfunction, and cancer therapy. In postmenopausal women, estrogen replacement therapy (2) was proven efficacious for the prevention of osteoporosis (3), and several lines of study suggested that 17\( \beta \)-estradiol (E\(_2\)) has beneficial effects in cardiovascular (4, 5) and selected neurodegenerative diseases (6). Unfortunately, the prolonged use of this hormone has been associated with increased risk of breast and uterine cancer (7). The discovery that synthetic ligands of the ER may exhibit tissue-specific agonist or antagonist activity raised a new interest in the use of these compounds for estrogen replacement therapy (8, 9). These selective estrogen receptor modulators or SERMs are identified by comparative screening in cells of different origin to characterize their tissue-specific profile (agonist/antagonist). Generally, the study is carried out in transformed cell lines stably or transiently transfected with ER\(_{\alpha}\) or -\( \beta \) and a reporter of the receptor’s activated state. In addition to limiting the analysis to a selected number of cells, this method may also provide erroneous or defective results. In fact, the tissue-specific agonist/antagonist activity of SERMs has been attributed to the presence
of cell-specific proteins capable of interacting with the hormone receptor complex (10), and these proteins may be aberrantly expressed in cancer cells (11). Thus, the major shortcoming of this screening procedure is associated with the requirement of further in vivo analysis for the identification of the pharmacodynamic properties of the molecule to be developed. The availability of an engineered mouse carrying an ER reporter expressed ubiquitously as a transgene would represent a remarkable advancement for the identification and profiling of new SERMs. In addition, such a model would be invaluable for the spatio-temporal localization of ER activity and could provide data of major impact for the full comprehension of estrogens and ER functions from development to aging. Such an experimental system can hardly be generated by classical transgenesis because of the difficulty in obtaining a regulated expression of the transgene (12). To overcome this limitation, we made use of insulator sequences previously described to oppose the interference of the host genome on the expression of the ectopic genes (13, 14).

In this study we describe a construct that led to ubiquitous and estrogen-regulated expression of a reporter transgene. The transgenic mouse we generated represents an innovative model for the study of the in vivo dynamics of intracellular receptor activity.

RESULTS

Generation of the Constructs and Their Preliminary Analysis in Stably Transfected Cells

The choice of luciferase as reporter gene was dictated by several factors: 1) no protein structurally related to this enzyme has been described in mammals; 2) the assay for the quantitation of this enzymatic activity in tissue homogenates is extremely sensitive; 3) very efficient antibodies are available for the localization of the protein by immunohistochemistry. To obtain a minimal constitutive and strong estrogen-inducible expression of the reporter, the arrangement of the promoter cassettes was selected experimentally by transient transfection studies in MCF-7, SK-N-BE, and HeLa cell lines (not shown). A number of constructs containing different deletion mutants of the minimal promoter from the tk gene combined to different synthetic multimers of the canonical estrogen-responsive element (ERE), were assayed. The best arrangement found consists of two palindromic EREs spaced 8 bp apart located at 55 bp upstream from the tk promoter. To limit position effects and gradual extinction of the reporter expression (12), we generated constructs in which the transgene was flanked by either the insulators MAR (matrix attachment region) (15) or HS4 (β-globin hypersensitive site 4) (16) (Fig. 1A). The efficiency of these boundary elements was tested by stable cotransfection of the constructs generated, and the pSV2Neo vector in the ERα-positive MCF-7. 48 clones for each construct were isolated, expanded, and tested for luciferase expression in the presence or absence of 1 nM E2 (Fig. 1B). In the absence of hormone, luciferase activity could be measured in 77% (37/48) and 40% (19/48) of the clones transfected with pHS4 and pMAR, respectively. In about 80% of these (inducible clones), 16 h of E2 treatment caused a significant increase in the reporter activity (at least 3-fold over basal levels). When pERE was transfected, basal luciferase activity could be detected only in 19% (9/48) of the clones, and in 44% of these the enzymatic activity was E2 inducible. Next, we evaluated the relationship between the number of copies integrated and luciferase expression in the presence or absence of 1 nM E2 (Fig. 1B). In the absence of hormone, luciferase activity could be measured in 77% (37/48) and 40% (19/48) of the cells transfected with pHS4 and pMAR, respectively. In about 80% of these (inducible clones), 16 h of E2 treatment caused a significant increase in the reporter activity (at least 3-fold over basal levels). When pERE was transfected, basal luciferase activity could be detected only in 19% (9/48) of the clones, and in 44% of these the enzymatic activity was E2 inducible. Next, we evaluated the relationship between the number of copies integrated and luciferase expression in the absence or presence of E2. In the absence of E2, linear correlation analysis of the two variables produced lines of best fit with an r coefficient of 0.66 for pHS4 and 0.79 for pMAR-transfected clones. After 16 h of E2 induction, the r calculated was 0.47 and 0.54 for the two groups of clones. These values of r indicate a positive correlation between the two variables analyzed, even though

Fig. 1. Insulator Activity in Stably Transfected MCF-7 Cells

A. Vectors used for the generation of stably transfected cells. B. MCF-7 cells were cotransfected with pSV2Neo and the indicated constructs. After selection, 48 single clones for each transfected plasmid were isolated and expanded. Luciferase activity was measured in the absence or presence of 1 nM E2 for 16 h. Bars represent the percentage/total of clones expressing detectable amounts of luciferase (upper graph) or responsive to the hormonal treatment with at least a 3-fold increase of luciferase activity over basal (lower graph). The luciferase enzymatic activity was detected in two separate experiments after triplicate treatment.
these data most likely underestimate the insulator activity because the clones in which transgene rearrangements had occurred were not eliminated from the analysis.

These results are in agreement with previous studies showing that insulators confer a copy dependency of the transgene expression. In addition, here we show that these sequences considerably facilitate the estrogen-regulated expression of the transgene in the chromosomal context.

**Effect of Insulators on Estrogen-Dependent Transcription of the Reporter Gene in the Mouse**

Linearized pMAR and pHs4 vectors deleted of plasmid sequences were microinjected into oocytes explanted from C57Bl/6xDBA/2 F2 of mice zygotes. This outbred strain was chosen to ensure a high efficiency of transgenesis (17); furthermore, the presence of C57BL/6 in the genetic background confers a good responsiveness to estrogens (18, 19). Seventeen independent lines were obtained, but only 12 of these were fertile. 9 carrying the pMAR and 3 carrying the pHs4 construct. An initial screening for assessing basal and estrogen-inducible expression of the luciferase reporter was done by measuring the reporter enzymatic activity in tissue homogenates from ovariectomized mice of the F1 generation. Five organs were initially taken into consideration: uterus, liver and brain as well known targets for the hormone, and lung and heart as negative controls. Table 1 shows that among the lines that integrated the MAR transgene, three showed an estrogen-inducible expression of the reporter in uterus, brain, liver, and lung. In line 31 the hormone-inducible expression of the reporter was found in uterus, liver, and brain, while in lines 56 and 59 it was restricted to brain. We did not detect any basal or estrogen-inducible luciferase activity in the heart. In lines 13 and 77, basal expression of the reporter is low; however, treatment with E2 did not result in its increase. In transgenic mice carrying the pHs4 construct, we observed very little expression of the reporter in the organs investigated; only line 61 showed low basal and E2-induced expression of luciferase.

Considering that minimal promoters are heavily influenced by position effects, we observed ectopic expression of luciferase in only a few lines of mice; we concluded that the presence of insulators allows the position effects to be overcome without interfering with the hormone-regulated expression of the transgene.

**Characterization of Estrogen-Dependent Luciferase Expression in Transgenic Mice**

A further characterization of the activity of the transgene was carried out in line 2. Luciferase activity was measured in 26 different tissues from 2-month-old female mice, which had been ovariectomized 2 weeks before the experiment. To verify the capability of E2 to induce the transgene transcription, mice were treated for 16 h with either vehicle or E2 subcutaneously. Figure 2A shows that in the absence of hormonal stimulation a considerable level of luciferase expression was found in tissues such as bone marrow, brain, pituitary, liver, tongue, and mammary gland, while in others the enzymatic activity found was low, at the limit of detection. The hormonal treatment induced an increase of the enzyme content higher than 5-fold with respect to controls in liver, lung, spleen, bone marrow, brain, and thymus. In eye, uterus, bladder, skin, adipocyte, and spinal cord, the hormonal treatment resulted in an accumulation of luciferase less remarkable (between 2.5- and 4.9-fold over controls), but still clearly visible. Finally, the treatment did not result in any change in pancreas, tail, aorta, esophagus, thyroid, stomach, blood, tongue, skeletal muscle, or heart (Fig. 2B).

When compared with the distribution of ERα and -β, the distribution of luciferase activity indicated a strict correlation between E2 responsiveness and presence of the hormone receptors. Interestingly, the lung, which was originally taken as a control ER-negative organ, showed a high responsiveness to the hormonal treatment. This finding is in line with the recent report on the high content of ERβ in lung (20).

Adjacent slices were stained with antibodies raised against ERα or luciferase (Fig. 3). ERα immunoreactivity was clearly detected in nuclei of cells in stroma, endometrium, and glandular epithelium. Cytoplasmic staining of luciferase was clearly visible in the same cell types. In both cases, no staining was detected when preimmune serum was used.

### Table 1. Luciferase Expression in Transgenic Lines

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<td>Heart</td>
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−, Below limits of detection; n.i., expressed, not inducible; +/−, low level of expression, inducible; +, high level of expression, inducible.
Bone Marrow Cells in Primary Culture Retain the Estrogen-Inducible Luciferase Expression

Initial pharmacological characterization of the luciferase expression was done in primary cultures of bone marrow (Fig. 4). The cells were treated for 16 h with increasing concentrations of E2 (0.01–10 nM) or with 100 nM of two ER antagonists: 4-hydroxytamoxifen (T) and ICI 182,780 (ICI) alone or in the presence of 1 nM E2. E2 induced a dose-dependent increase of luciferase accumulation blocked by the presence of ICI 182,780. ICI 182,780 by itself did not produce any effect. Conversely, 4-hydroxytamoxifen induced a significant increase of luciferase levels even though lower than E2 at the same concentration. In coadministration with E2, 4-hydroxytamoxifen induced higher luciferase accumulation, yet the level reached was still lower than with E2 alone. This is compatible with the partial agonist activity of 4-hydroxytamoxifen and with the fact that it is present in the solution at a concentration 100-fold higher than E2. As control, we also tested progesterone and dexamethasone (10 nM). Neither ligand had any effect on the ER reporter (Fig. 4).

Taken together, these data confirm that, even in cells explanted from engineered mice, the transgene is controlled by ligands of ER with modalities recapitulating those reported for the natural target genes.

Pharmacological Modulation of Luciferase Expression in Vivo

Two-month-old male mice were injected s.c. with 50 μg E2/kg and killed after 3, 6, or 16 h. As shown in Fig. 5 (upper panel) the maximal luciferase accumulation was observed at 6 h after treatment both in liver and bone tissues. When mice were treated for 6 h with increasing concentrations of the hormone (Fig. 5, middle panel), the maximal effect on luciferase activity was detected at 50 μg/kg. Interestingly, the administration of 250 μg E2/kg induced, in the bone, a luciferase accumulation lower than with 50 μg/kg. Thus, the luciferase accumulation is time and dose dependent. Next, the effect of in vivo administration of the two ER antagonists was investigated. Figure 5 (lower panel) shows that the s.c administration of 250 μg tamoxifen/kg for 6 h increased the level of luciferase in liver and bone 12 times and 7 times, respectively, confirming also in vivo the partial agonist activity of tamoxifen in these tissues. The injection of 250 μg tamoxifen/kg or ICI 182,780, 1 h before the administration of 50 μg E2/kg, inhibited the E2-dependent activation of luciferase expression as expected from the antagonist effect of these compounds with respect to E2.

DISCUSSION

We generated a transgenic mouse model for the study of the dynamics of ER transcriptional activation in primary tissue cultures and in vivo. Several lines of evidence indicate that the model generated fulfills its purpose: 1) E2 administration results in accumulation of luciferase in organs and tissues reported to express either or both of the two ER isoforms; 2) in uterus, immunohistochemistry shows colocalization of lucif-
erase and ER immunoreactivity; 3) experiments in primary cultures of bone marrow show that luciferase activity is controlled by E2 in a dose-dependent fashion (with highest E2 activity compatible with its affinity for the receptors) and ER antagonists display a profile of activity in line with previous reports in vivo and in vitro (21); 4) experiments in vivo show the dose- and time dependency of E2 activity, the antagonist activity of ICI 182,780, and the partial agonist activity of tamoxifen in bone and liver.

We believe that the key to the realization of our model was the use of insulators. It is, in fact, well known that the expression of transgenes driven by weak promoters is heavily influenced by enhancers/silencers surrounding the regions of insertion; in addition, methylation may gradually extinguish their transcriptional activity. In the past, insulators have been successfully used to counteract these effects in specific tissues. Here, we demonstrate that their use can be extended to the achievement of the ubiquitous and regulated expression of a given gene. Sixty percent of the transgenic lines obtained expressed luciferase in an estrogen-dependent fashion at least in some organ.

In-depth analysis of one positive line showed that in 26 target tissues, the expression of the transgene is correctly regulated after in vivo administration of E2. Yet, in 40% of the mouse lines developed, the expression of luciferase was either undetectable or not modulated by E2.

This could be due to the use of a weak promoter, which might have slightly restricted the possibility of reaching detectable levels of reporter expression. The E2-independent expression of luciferase may be ascribed to rearrangements of the vector during the integration in the mouse genome. Indeed, also the study of stably transfected MCF-7 cells showed that in about 20% of the clones the expression of luciferase was insensitive to the presence of E2.

The system generated represents a major advancement for the understanding of the physiology of compounds active through the ERs. In the last decade, the ability to transfect cells in culture with reporters of ER transcriptional activity granted a novel insight into the complexity of estrogen action. It was shown that the binding of the hormone-receptor complex to the specific sequences of the promoter is not sufficient to
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Two million bone marrow cells were suspended in phenol red-free RPMI 1640 with 10% stripped serum. Cells were treated with increasing concentrations of E2 (0.001, 0.01, 1.0, and 10 nM) or with 100 nM ICI 182,780 (ICI) or 4-hydroxytamoxifen (T) alone or with 1 nM E2. Progesterone (Prog) and dexamethasone (Dex) were used at 10 nM final concentration. Control cells (C) were treated with the same concentration of ethanol present in the hormone solutions (0.0001%). Bars represent the average ± SEM of five individual experiments each done in triplicate. *, P < 0.01 as compared with the control; **, P < 0.005 as compared with the control; ⋄, P < 0.05 as compared with the T-treated; P values were calculated with ANOVA followed by Scheffé test.

ensure the hormone-regulated transcription of the target genes. The ER must, in fact, interact with a series of proteins modulating its transcriptional activity (22, 23). These findings were supported by crystallographic studies showing the structural conformation of ER bound to natural or synthetic ligands (24, 25). These and other investigations on steroid receptors demonstrated how synthetic ligands, by inducing specific structural conformations that modify the possibilities of the receptor to interact with its coregulators, may change its transcriptional activity in a tissue-specific fashion (10, 23, 26). In addition, several studies underlined that the binding of the specific hormone is not indispensable to ER transcriptional activation. Unliganded ER was shown to regulate the transcription of target genes after activation of specific kinases (27, 28). Finally, ER dosage may also constitute an important element in the control of ER tissue-specific activities.

A major challenge at present is to demonstrate how these mechanisms are relevant in physiological systems and how ER activity is regulated in its numerous target cells. The model generated will facilitate these studies by providing a system in which the activity of the receptor on ERE-containing genes can be assessed in a very restricted time frame. To this aim, we purposely made use of the natural firefly luciferase gene, the turnover of which in mammalian cells is about 3 h (29). By measuring the levels of luciferase accumulation, therefore, we will be able to monitor the state of activity of the receptor in response to the fluctuating hormone levels during the estrous cycle or after administration of ER ligands. In addition, these mice will allow identification of novel tissues and cell types targeted for the hormone in vivo in both sexes.

Further investigation is necessary to understand whether the high content of luciferase in bone marrow, brain, tail, tongue, or liver observed in this study should be ascribed to the tissue characteristics facilitating the recovery/measurement of the luciferase enzymatic activity, to the low catabolism of the exogenous protein, or to the activation of the unliganded resident receptor via cross-coupling with membrane receptors. We would rule out the possibility of luciferase induction by ERR (ER-related receptor) orphan receptors based on the observation that tissues such as kidney and heart, known to express very high concentrations of ERRα and γ, display a very low basal activity of the reporter (30–32). Similarly, a more accurate evaluation of the time course of E2 induction is necessary before drawing any conclusion on the potency of the hormonal treatment in the various organs. The present study was carried out at 16 h of hormonal treatment. It is likely that the relatively low E2-dependent accumulation of luciferase that we observed in certain organs (e.g., uterus, mammary glands) is due to ER down-regulation, which in these organs occurs in a few hours after E2 administration. Appropriate time-course studies will better clarify the kinetics of ER activity in the various tissues.

From the pharmacological point of view, the system generated is very interesting, particularly for the identification of novel SERMs because it will identify in which organs the molecule of interest displays full, partial agonist, or antagonist activity. The preliminary assessment of the activity of 4-hydroxytamoxifen in bone marrow cells and in vivo, supports the validity of this model in this type of studies. In previous studies, reporter-based systems for the in vivo identification of ligands for intracellular receptors were generated using fusion proteins between the RXR and RAR ligand binding domain and the DNA binding domain of the yeast protein GAL4 (33, 34). These systems were proved to be useful for the detection of endogenous ligands; however, because of the relevance of protein/protein interaction in the activity of intracellular receptors, GAL4-receptors fusion products might be unable to undergo conformational changes indispensable for the action of tissue selective synthetic ligands (10, 23, 26).

In spite of the fact that the system generated will not provide an insight on the exact nature of the ER activated [ERα, ERβ, or other proteins not known active through estrogen response elements (EREs)], appropriate breeding of the ER transgenic mice with selective ERα and -β knockout (K.O.) mice will erase this limitation.

The transgenic mouse generated in this study can be used to produce models for the physiological, pharmacological, and toxicological analysis of other intracellular receptors. In addition, because of the intrinsic characteristics of the reporter above specified,
the model could be particularly suited for studying the pharmacokinetic profile of natural and synthetic ER ligands. Finally, these transgenic mice can be used as biosensors to investigate whether environmental or food pollutants act as endocrine disruptors by interfering with the physiological state of ER activity.

MATERIALS AND METHODS

Plasmid Construction

Each functional cassette of the vector used for transgenesis was flanked with unique restriction sites to facilitate further manipulations. Each element of the construct generated was sequentially cloned in the vector pBluescript (Stratagene, La Jolla, CA). The basic construct without insulators was named pERE and contains two canonical EREs (35) (ERE2X) spaced by 8 bp, a minimal thymidine kinase (tk) promoter from herpes simplex virus (36) (55 bp downstream from the ERE2X) and the luciferase reporter gene (Fig. 1A). This construct was assembled with the following components: 1) the 2,731-bp DNA fragment encoding the luciferase excised from the pGL2basic vector (Promega Corp., Madison, WI) with the SalI restriction enzyme blunted and ligated into the blunted HindIII site of pBluescript; 2) the 168-bp BamHI/XhoI fragment containing the tk promoter from pBLCAT2 (37), blunted and ligated into the blunted PstI site of the pBluescript; 3) the 82-bp XhoI/ClaI fragment containing the ERE2X excised from pGL2basic vector (Promega Corp.) in which it has been previously cloned (see below), blunted, and ligated into the blunted SalI site of the pBluescript.

Two tandem copies of the insulators HS4 (2.4-kb DNA fragment) from chicken β-globin gene were obtained by digesting the vector pBS(II)HS4, generously provided by S. Y. Tsai (38), with SalI restriction enzyme; a single copy of MAR (3-kb DNA fragment) from chicken lysozyme gene was excised with digestion of the pBSKMAR, kindly provided by L. Hennighausen (39), by XbaI/BamHI restriction enzymes. The insulator fragments were blunted and inserted in the blunted KpnI and NotI sites located at the 5′- and 3′-end of pERE.
estrogen receptor pharmacology

**Generation of ERE2X**

The two oligonucleotides, 5'-GATCCGGACGTCACGATGAC
CTAGT-3' and 5'-GATCTAGGTCAGTGACCTGGC-3', were annealed, the resulting double strand oligo was ligated and digested with BamHI, and the bands corresponding to monomers or multimers were extracted from an acrylamide gel as described previously (40) and ligated into the BglII site of pGL2 basic vector.

**Cell Cultures and Transfections**

Breast carcinoma MCF-7, neuroblastoma SK-N-BE, and cervix carcinoma HeLa cell lines were routinely grown in RPMI 1640 medium supplemented with 10% FBS. Stable transfections of MCF-7 cells were performed with the calcium phosphate procedure as previously described (41). Twenty four hours before transfection, 1.5 x 10^6 cells were seeded in Petri dishes with RPMI 1640 supplemented with 10% FCS; 6 h before addition of 1 ml CaPO4/DNA mixture the medium was replaced with DMEM supplemented with 10% FCS. The CaPO4/DNA mixture used for transfection contained 1 mg of pMAR or pMH vectors and 9 μg salmon sperm DNA. Forty eight hours after transfection, 300 μg/ml G418 (Life Technologies, Inc.) were added to the culture medium. Medium and selective agents were replaced three times a week. After 21 days selection, 48 clones for each transfection were isolated with cloning rings

**Transgenic Mice**

For microinjection, linearized pMAR and pHS4 constructs were dephosphorylated with pronuclear DNA injection of zygotes C57Bl/6xDBA/2, F2 generation, using previously described (27), and the enzymatic assay was carried out as described in detail below.

**Luciferase Enzymatic Assay**

Luciferase enzymatic activity in the cell and tissue extracts was measured by a commercial kit (luciferase assay system, Promega Corp.) according to the supplier indications. The light intensity was measured with a luminometer (Lumat LB 9501/16, Berthold, Wildbad, Germany) over 10 sec and expressed as relative light units (RLU) over 10 sec/μg proteins.

**Immunohistochemistry**

Uteri of ovariectomized mice, treated as before, were dissected and fixed through immersion in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 (PB), for 5 h. Tissues were dehydrated with an ascending ethanol scale, clarified with xylene, and processed for paraffin embedding. Serial 4 μm microtome sections were cut and collected onto slides coated with polyclonal antibodies for 16 h drying at 37 °C, sections were hydrated through a descending ethanol scale and boiled in 10 mM citrate buffer (pH 6.0) for 15 min in a microwave oven, washed for 10 min with PBS, and then processed for luciferase and ERα immunodetection at room temperature. Sections were first incubated for 30 min with 0.3% H2O2 to quench endogenous peroxidase activity and subsequently washed three times with PBS for 10 min. After saturation with 10% preimmune goat serum supplemented with 0.3% Tween 20 (Sigma), sections were incubated with the antiluciferase (Sigma, 1:1800 dilution in PBS with 10% goat serum and 0.3% Tween 20) polyclonal antibodies for 16 h, washed with PBS (six times, 10 min each), incubated for 60 min with an anti-rabbit secondary antibody (raised in goat, 1:200 dilution in PBS supplemented by 1% goat serum and 0.3% Tween 20; Vector Laboratories, Inc., Burlingame, CA) and then washed again (six times with PBS, 10 min each). Antibody-antigen detection was obtained by 40 min incubation with avidin-biotin-horseradish peroxidase (HRP) from an ABC kit (Vector Laboratories, Inc.). Immunostaining was visualized by exposure to HRP substrate 3,3′-diaminobenzidine (DAB Fast Tablet Set, Sigma). After one wash in PBS and few tap water changes, sections were allowed to air dry and then covered. Pictures were taken with a digital camera (Coolpix 990; Nikon, Melville, NY) applied to a Axioscope microscope (Carl Zeiss, Thornwood, NY).

**Primary Bone Marrow Culture**

After mice were killed, bone marrow cells were flushed out from femur and tibia of ovariectomized animals, using a syringe filled with PBS. Cells were collected in a 15 ml Falcon tube (Becton Dickinson and Co., Meylan Cedex, France) and washed once with PBS. After centrifugation the cell pellet
was resuspended in RPMI 1640 supplemented with 10% DCC-FBS; cells were counted and plated in a six-well dish (2 × 10^6 cells per well). For the treatment, all compounds were dissolved in ethanol and added to the medium at the indicated concentration. After 16 h the cells were collected in Eppendorf tubes, washed once with PBS, re-suspended in cated concentration. After 16 h the cells were collected in dissolved in ethanol and added to the medium at the indi-

**Experimental Animals**

Animal experiments performed in this study were conducted according to the “Guidelines for Care and Use of Experimental Animals.”

**Acknowledgments**

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Erratum

In the article “Pleiotropic Effects of Substitutions of a Highly Conserved Leucine Residue in Transmembrane Helix III of the Human Lutropin/Choriogonadotropin Receptor with Respect to Constitutive Activation and Hormone Responsiveness” by Hiromitsu Shinozaki, Francesca Fanelli, Xuebo Liu, Julie Jaquette, Kazuto Nakamura, and Deborah L. Segaloff (Molecular Endocrinology 15: 972–984, 2001), The heading for the right column of Table 1 should read “125I-hCG Bound (ng/10⁶ cells)”, not “Kₐ (nM)”.

Estrogen Receptor Pharmacology in Vivo