Sustained mammary gland-directed, ponasterone A-inducible expression in transgenic mice

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ABSTRACT **The ability to regulate temporal- and spatial-specific expression of target genes in transgenic mice will facilitate analysis of gene function and enable the generation of murine models of human diseases. The genetic analysis of mammary gland tumorigenesis requires the development of mammary gland-specific transgenics, which are tightly regulated throughout the adult mammary epithelium. Analysis of genes implicated in mammary gland tumorigenesis has been hampered by mosaic transgene expression and the findings that homozygous deletion of several candidate genes (*cyclin D1*, *Stat5A*, *prolactin receptor*) abrogates normal mammary gland development. We describe the development of transgenic mouse lines in which sustained transgene expression was inducibly regulated, both specifically and homogeneously, in the adult mammary gland epithelium. Transgenes encoding RXRα and a chimeric ecdysone receptor under control of a modified MMTV-LTR, which targets mammary gland expression, were used. These transgenic 'receptor' lines were crossed with transgenic 'enhancer' lines in which the ecdysone/RXR binding site induced ligand-dependent expression of transgenic β-galactosidase. Pharmacokinetic analysis of a highly bioactive ligand (ponasterone A), identified through screening ecdysteroids from local plants, demonstrated sustained release and transgene expression *in vivo*. This transgenic model with both tightly regulated and homogeneous transgene expression, which was sustained *in vivo* using ligands readily extracted from local flora, has broad practical applicability for genetic analysis of mammary gland disease.—Albanese, C., Reutens, A. T., Bouzahzah, B., Fu, M., D'amico, M., Link, T., Nicholson, R., Depinho, R. A., Pestell, R. G. Sustained mammary gland-directed, ponasterone A-inducible expression in transgenic mice. *FASEB J.* 14, 877–884 (2000)**

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THE ABILITY TO modulate gene expression *in vitro* and *in vivo* has become a powerful tool in understanding the role(s) of specific gene products in the normal growth and development of cells, tissues, and, through transgenesis, in the whole animal. The utility of tissue-specific transgenic models to examine the function of a target gene is limited by several practical considerations. The characteristics inherent to an ideal inducible transgenic system include low basal level expression, high inducibility, tissue-specific targeting, and sustained induction. The fidelity of these characteristics is particularly important in the delivery of toxic or lethal genes and for the delivery of CRE recombinase to allow efficient somatic excision (1). Binary systems, in which one mouse line contains an activator of expression and the second mouse line contains the silenced gene of interest, have been developed to examine the expression of potentially lethal or hypomorphic genes. Furthermore, inasmuch as the acquisition of somatic mutations in the adult contribute to the onset and progression of tumorigenesis, genetic manipulation postnatally in a defined tissue is likely to recapitulate oncogenic mechanisms more faithfully and thus provide a more informative model for the study of disease.

Transgenic approaches to controlled inducible misexpression of target genes have used transactivators such as the VP16 and Gal4/UAS activation system driven by tissue-specific promoters (2, 3). The tetracycline-regulated system was developed to provide temporal and spatial control of gene expression (4, 5). In the presence of the ligand, the transactivator tTA is bound to the tetO promoter, and gene activity is silent until the ligand is removed (4, 5). Alternatively, in the reverse tTA system, transcrip-

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tional activity is induced by the addition of ligand. Successful application of this system to transgenics (6) has been balanced by reports of mosaic induction, background leakiness, and poor expression of the transactivator, perhaps related in part to the inefficient processing of the *tetR* gene in mammalian cells, which may limit the utility of this approach for the study of cancer (1). Steroid hormone inducible systems have centered on the expression of chimeric receptors that bind nontoxic hormone ligands. Estrogen receptor chimeric transgenics induced by tamoxifen show promise (7), but evidence of mosaic transgene expression in target tissues (8) and the need to use toxic and teratogenic doses for robust expression (9) suggest that components of this system require further modifications.

The ligand for the 'ecdysone' system is a molting hormone, 20 OH-ecdysone, which normally binds a heterodimer of the *Drosophila* ecdysone receptor (EcR) and the product of the *ultraspiracle* gene in the context of a specific DNA binding site (10) (11). Ecdysteroids are neither teratogenic nor known to affect mammalian physiology. A chimeric receptor consisting of the VP16 transactivation domain fused to an amino-terminal truncation of the modified EcR (VgEcR) was shown to transactivate a reporter gene consisting of multimeric ecdysone enhancer binding sites with higher inducibility than comparable tet-regulated reporters (12). Furthermore, the basal level activity of the enhancer was low in mammalian cells, consistent with the potential utility of this system for transgenesis (12). Despite these promising features, sustained targeted transgene expression has not been reported, perhaps related to the lack of published pharmacokinetic data, tissue distribution analysis, and the unavailability of inexpensive ligands (1). In the current studies we describe the development and successful implementation of a system for targeted mammary gland-specific expression in transgenic mice, the purification of ponasterone A, a potent ecdysteroid ligand, from plants, and the use of sustained release ponasterone A.

MATERIALS AND METHODS

Plasmids and transgenic lines

The luciferase reporter $(E/GRE)_6$TK81LUC consists of multimeric E/GRE binding sites from $(E/GRE)_4$ ΔMTVLuc (12) linked to the minimal TK promoter in the pA_3LUC reporter plasmid (13). The modified MMTV promoter from MMTVp206 (14, 15) that incorporates the 5′UTR of v-Ha-ras was inserted into a vector containing a modified ecdysone receptor VgEcR (a gift from Dr. R. Evans) to form MMTV-VgEcR. The β-galactosidase cDNA, a derivative of pPD16.51, with a eukaryotic polyadenylation site (16) (a gift from Dr. A. Fire), was inserted into the EcR target plasmid $(E/GRE)_3$ΔMTV (from Dr. R. Evans). DNA constructions were prepared and injected into CBA/C57Bl6 fertilized eggs. The MMTV-VgEcR and CMV-RXRα constructions were coinjected. Genotyping was performed by genomic Southern blot analysis. Transgenic lines were bred into the FVB genetic strain of mice for at least four generations. The reporter line $(E/GRE)_3$β-Gal was bred to the receptor line and the presence of the three transgenes was confirmed by genomic Southern analysis. In addition, a distinct plasmid, pVgRXR, was obtained from InVitrogen (Carlsbad, Calif.) and used in cell culture experiments as indicated in the text.

Cell culture, DNA transfection, and luciferase assays

Cell culture, DNA transfection, and luciferase assays were performed as described previously (13). 293T (BOSC) cells (from Dr. D. Baltimore) were maintained in Dulbecco's modified Eagles medium with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were transfected by calcium phosphate precipitation with pVgEcR/RXRα and $(E/GRE)_6$TKLUC. Six hours after addition of the precipitate, the media were changed and treatment was performed for the time points indicated. At least two different plasmid preparations of each construct were used. Luciferase assays were performed at room temperature using an Autolumat LB 953 (EG&G Berthold). Luciferase content was measured by calculating the light emitted during the initial 10 s of the reaction and the values are expressed in arbitrary light units. Statistical analysis was performed using the Mann Whitney *U* test.

Ponasterone purification

The leaves of candidate species from the *Podocarpus* or *Taxus* genus of evergreen plants were ground in liquid nitrogen, refluxed in methanol, and subjected to high-pressure liquid chromatography (HPLC) over a micro-Bondpak C-18 column (3.9×300 mm column, Waters Inc., Milford, Mass.). Fractions (20 ml) of a 15–50% acetonitrile gradient were collected and lyophilized. The dried samples were resuspended in 100% ethanol and stored at −20°C. Ponasterone A was extracted from mammary tissue from ponasterone A-treated mice by grinding the mammary tissue in liquid nitrogen, refluxing overnight in ethanol and centrifuging at 5000 *g* for 20 min. The supernatant was dried and subjected to HPLC as stated above (17). In addition, purified ponasterone was obtained from Dr. K. Nakanishi and was used as indicated. Delayed release ponasterone A and placebo pellets were made by Innovative Research (Innovative Research of America, Sarasota, Fla.).

Immunohistochemistry and β-galactosidase assays

Mammary tissue was taken from lactating mice 1 day postpartum. For β-galactosidase staining, 5 mm cubes of tissue were fixed in 2% paraformaldehyde/0.02% glutaraldehyde for 1 h, washed with phosphate-buffered saline (PBS) twice, and stained at 30°C with 0.1% 4-chloro-5-bromo-3 indol-b-D-pyranoside in 2 mM $MgCl_2$, 5 mM EGTA, 0.02% (v/v) Nonidet P-40, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6{\cdot}H_20$ for 28 h (18). The samples were embedded in paraffin, counterstained with nuclear fast red, and examined for blue nuclei. For immunohistochemistry, samples were frozen and sectioned on a cryostat. Sections were fixed in cold acetone, treated with hydrogen peroxide, and incubated in either anti-VP16 primary antibody (V-20; Santa Cruz Biotechnology, Santa Cruz, Calif.) or anti-RXRα antibody (D-20; Santa Cruz) at a 1:2000 dilution overnight at 4°C. The samples were washed with PBS and incubated with a biotinylated secondary antibody (Santa Cruz) at a 1:1000 dilution for 30 min at room

temperature. The sections were incubated for 30 min at room temperature in avidin-horseradish peroxidase and stained with diaminobenzadine.

Reagents

20 OH-ecdysone was a gift from Dr. Peter Cherbas. Inokosterone and ponasterone A were gifts from Dr. Koji Nakanishi. Muristerone was obtained from Sigma (St. Louis, Mo); chemicals were obtained from Fisher (Fairlawn, N.J.) and were of HPLC grade.

RESULTS

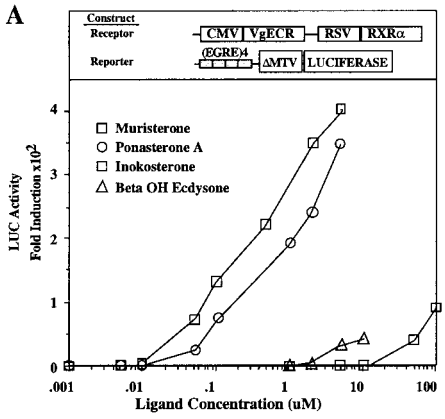
Identification of bioactive ecdysteroids

Previous analysis of ecdysteroid bioactivity using morphological transformation of *Drosophila* Kc167 cells indicated phytoecdysteroids and their derivatives such as inokosterone functioned as potent ecdysteroids (19). In previous studies, a chimeric ecdysone receptor, in which the amino-terminal truncation of a modified EcR was attached to the VP16 activation domain (VgEcR), was shown in reporter assays to enhance ecdysteroid-induced reporter activity compared with the parental EcR receptor (12). Comparison was therefore made of the relative activity of these ecdysteroids in a synthetic luciferase reporter assay driven by a multimeric optimized enhancer element for the modified chimeric VgEcR/RXRα receptor. Dose response curves were conducted with each ligand on cells transfected with the reporter gene and an expression plasmid for the bi-cistronic VgEcR/RXRα receptor, and the relative activity was compared with vehicle (**Fig. 1*A***). Analyses for basal and ligand-induced activity performed after 24 h of treatment showed that ponasterone A is ~1000-fold more active than 20 OH-ecdysone. Ponasterone A and muristerone exhibited similar potencies (Fig. 1*A*). Basal activity of the reporter was inhibited by receptor in the absence of ligand by 40% (data not shown) and no transcriptional activation was observed in the absence of cotransfected RXRα (data not shown).

To identify a source of bioactive ponasterone A, candidate flora were screened (Fig. 1*B*). Ponasterone A was extracted as described previously (17). Activity of the extracted material was compared with the activities of purified ponasterone A. Relative abundance was expressed as milligrams of ponasterone A/kilogram of material. *Podocarpus macrophyllus* D. Don and *P. macrophyllus* v. Maki both produced ponasterone A as did *Taxus cuspidata, T. chinensis, T. canadasis,* and *T. x media 'Hicksii'* (Fig. 1*B*).

ponasterone A delivery to the mammary gland

As ponasterone A was the most bioactive ecdysteroid in tissue culture reporter assays, the *in vivo* serum and tissue half-life of ponasterone A activity was determined in the mouse. To improve sensitivity of the reporter system and accurately measure low ponasterone A concentrations, a reporter $(E/GRE)_6$TKLUC was made that enhanced sensitivity by fourfold (**Fig. 2*A***). Ponasterone A activity was assessed in serum and tissues using this reporter. Serum samples from the mouse were assayed using the reporter gene with comparison made to a standard curve performed using purified ponasterone A (a gift from Dr. K. Nakanishi). Serum levels of ponasterone A were readily detectable 15 min after intraperitoneal (i.p.) injection (Fig. 2*B*). At least four different animals were compared at each time point. The half-life of serum ponasterone A activity,



B

Species with Ponasterone A

Name	Native Habitat	Relative Amount
P. macrophyllus var. Don	Japan	+ + + + +
P. reichei	Mexico	+ +
P. macrophyllus var. Maki	China	+
Taxus chinensis	China	+ +
Taxus canadensis	USA	+
Taxus x media 'Hicksii'	USA	+
Taxus cuspidata	Japan	+

Figure 1. Identification of a highly bioactive ecdysone analog from local flora. *A)* Determination of the potency of different ecdysteroids *in vitro.* 293T cells were transfected with the ecdysteroid reporter gene (E/GRE)ΔMTV LUC and the expression vector pVgEcR/RXRα. After transfection, the ecdysteroid ligands (in ethanol) were added to the media for 18 h. The extracts were assayed and -fold stimulation of luciferase activity was assessed. A comparison was made with ethanol-treated controls. *B)* The relative bioactivity of ponasterone A extracted from the leaves of various evergreen species. The samples were assayed as in panel *A*. +, 20–50 mg/kg, +++++, 220 mg/kg.

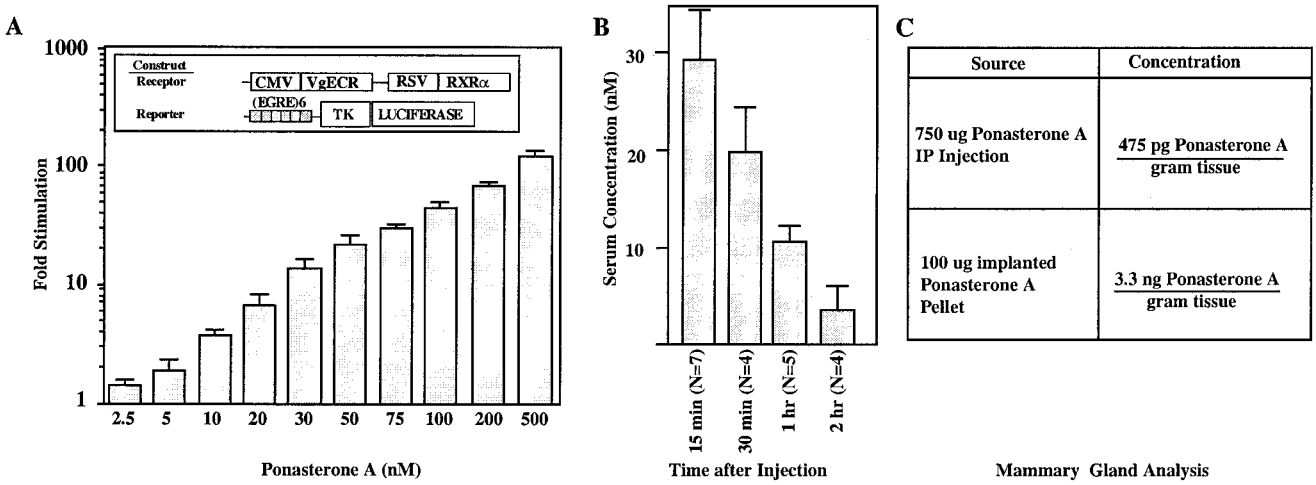


Figure 2. Ponasterone A is detectable in mammary tissue *in vivo. A)* Dose response curve of ponasterone A using the $(E/GRE)_6$TKLUC reporter in the *in vitro* bioassay. *B)* Pharmacokinetic analysis of adult female mice after i.p. injection with 750 μg ponasterone was suspended in 1:1(vol:vol) ethanol and Tween 80. The mice were bled at the times shown and the serum was assayed for ponasterone A activity using the *in vitro* assay in panel *A*. The data is mean -fold induction ± SE of N separate experiments as indicated in the figure. *C).* Ponasterone A concentration was determined in mammary tissue after either i.p. injection or implantation. HPLC fractionation of ponasterone A was performed on mammary tissue of adult female mice collected 1 day (injected mouse) or 21 days (implanted mouse) after administration of ponasterone A. The fraction containing ponasterone was assayed as in panel *A* and used to determine tissue ponasterone A concentration.

determined through sequential analysis after i.p. injection, demonstrated rapid clearance with an activity half-life of 48 min as determined by plotting the serum concentration vs. time on a semilogarithimic scale (20).

As the goal of the current studies was to direct mammary gland-specific transgene expression, the concentration of ponasterone A was determined in the mammary gland 24 h after i.p. injection of ponasterone A. HPLC purification of ponasterone A within the mammary gland was performed as described in Materials and Methods. The activity of the HPLC-purified material was then assessed using the $(E/GRE)_6$TKLUC reporter assay with comparison made to the standard curve. The ponasterone A concentrations were 400–500 pg/g of tissue (Fig. 2*C*). Sustained delivery of steroids through subcutaneous (s.c.) implantation has previously provided bioactive levels for treatment of steroid deficiency states in mice and humans (21). Serum ponasterone A activity was therefore assessed after s.c. implantation of ponasterone A pellets. Ponasterone A was incorporated into 21 day release implantation pellets and placed under the skin of mice as described in Materials and Methods. Blood was taken at days 4 and 15 postimplantation and assayed. Ponasterone A was detected in mammary tissue (Fig. 2*C*). In mammary gland tissue from mice carrying the 100 μg implant, ponasterone A concentrations were 3–4 ng/g of tissue. Ponasterone A activity was undetectable in peripheral blood (data not shown).

Mammary gland-targeted VgEcR/RXRα transgenic mice

Transgenic mice were generated in which the enhancer line expressed an ecdysone-responsive β-galactosidase reporter gene, $(E/GRE)_3\Delta$MTVβ-Gal, as a marker of ecdysteroid-regulated gene expression (**Fig. 3*A***). VgEcR was targeted to the mammary gland through the use of a modified MMTV promoter construction (MMTVp206) (15, 22), which enhances mammary gland-specific transgene expression through the addition of the 5′ UTR from v-Ha-ras (15). Three founder lines were established. The receptor and enhancer mice were bred to form triply transgenic reporter/ecdysone enhancer (TR/EE) lines and the integrity of the triply transgenic mice (TR/EE lines) was confirmed through genomic Southern analysis (Fig. 3*B, C*). The presence of the RXRα transgene in the TR/EE lines was confirmed by genomic Southern blot analysis (Fig. 3*B*, lane 2). The presence of the RXRα protein in the mammary gland tissue of the TR/EE lines was confirmed by Western blot analysis with a comparison made to the immunoreactivity of *in vitro* translated RXRα (right panel, Fig. 3*B*). The presence of RXRα in the mammary gland of the TR/EE lines was confirmed by immunohistochemistry using an RXRα-specific antibody as described in Materials and Methods (lower left panel, Fig. 3*B*). Minimal background staining of the tissue was observed in the absence of primary antibody (lower right panel, Fig. 3*B*).

The presence of the VgEcR transgene in the

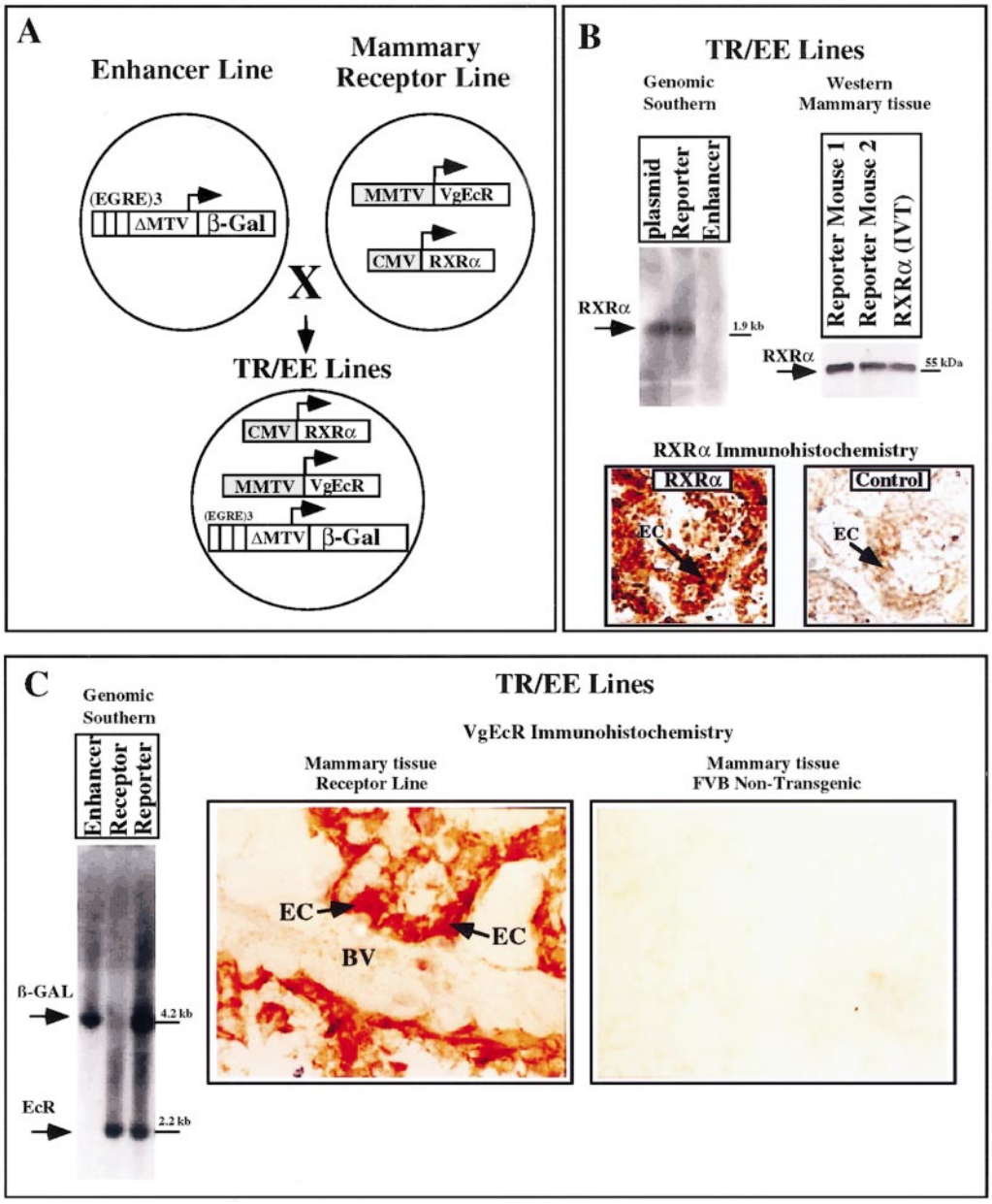


Figure 3. Generation of mammary tissue-specific ecdysone-inducible transgenic mouse lines. Schematic diagram of the three transgenic mouse lines (*A*). The β-galactosidase ('Enhancer') mouse 1 was generated by integration of the β-galactosidase cDNA inserted into an expression vector $(E/GRE)_3\Delta MTV$. The 'Receptor' mouse (mouse 2) contains both the RXRα cDNA driven by the CMVIE promoter and a chimeric ecdysone receptor (VgEcR) under control of the MMTV-LTR targeting promoter. The Reporter line, or tri-transgenic ecdysone enhancer line (TR/EE), incorporates all three transgenes (*B*). Upper panel, left: genomic Southern blot analysis probed with an RXRα probe. Lane 1, transgene plasmid DNA; lane 2, Reporter line; lane 3, Enhancer line. Upper panel, right: Western blot analysis for RXRα in mammary tissue from TR/EE mice. Lower panel, left: immunohistochemical staining for RXRα in mammary tissue where EC are endothelial cells; lower panel, right: the same tissue without the addition of primary antibody. *C)* Genomic Southern blot analysis for VgEcR and β-gal transgenes in either the 'Enhancer line', 'Receptor line', or TR/EE 'Reporter line'. Immunohistochemical staining was performed on mammary tissue using the VP16 antibody to the chimeric receptor (VgEcR) expressed in the mammary tissue as described in Materials and Methods. Magnification is 40×. The adjacent panel shows immunostaining of a section of mammary tissue from a nontransgenic control mouse under the same immunohistochemical conditions.

TR/EE lines was confirmed by genomic Southern analysis (Fig. 3*C*). The presence of the VgEcR receptor expression was assessed in the mammary gland tissue from lactating TR/EE lines using immunohistochemistry and a specific antibody to VP16 (Fig. 3*C*). Immunopositivity was observed throughout the mammary epithelium (depicted as EC in the left panel of Fig. 3*C*). No staining was observed within adjacent blood vessels (blood vessel (depicted as BV in the left panel of Fig. 3*C*). The mammary glands of nontransgenic control mice were immunonegative for the VgEcR (Fig. 3*C*, right panel). mRNA for the

VgEcR was detectable by Northern blot analysis in transgenic mammary gland but was not detectable in heart, liver, or salivary gland (data not shown).

To evaluate the tissue-specific expression and functional activity of the triply transgenic mice, the TR/EE mice were treated with ponasterone A and tissues were analyzed for β-galactosidase abundance after 24 h. Costaining with fast red was performed to identify nuclei. The basal level β-galactosidase abundance was undetectable in the mammary gland in the absence of ponasterone A (**Fig. 4*A***). Ponasterone A (750 μg) induced β-galactosidase activity uniformly throughout the mammary gland (Fig. 4*B*). Analyses performed of other tissues showed basal low but detectable levels of β-galactosidase activity in the salivary gland (Fig. 4*C*), but no activity in the liver, heart, spleen (Fig. 4*D–F*), kidney, ureter, ovary, or uterus (data not shown). The low level of expression within the salivary gland is consistent with previous reports that the MMTV promoter may direct expression to the salivary gland.

In many transgenic experiments, sustained expression may be required. Ponasterone A pellets were implanted s.c. in the subscapular region of the enhancer/receptor mice using a trocar according to the manufacturer's direction. β-Galactosidase staining of the mammary gland tissue performed after 10 days (**Fig. 5**) was observed to be uniform throughout the mammary epithelium. These studies demonstrate that sustained transgene expression can be maintained through ponasterone A implantation.

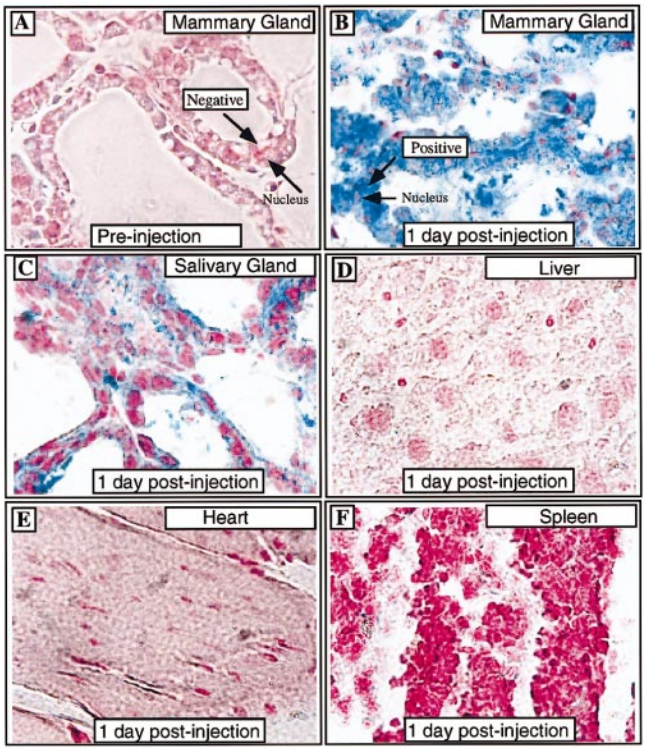


Figure 4. Mouse lines show mammary tissue-specific, inducible expression. *A)* Mammary tissues from the reporter mouse in the untreated state. (EC = epithelial cells) *B–F)* Tissues from the transgenic reporter mouse after i.p. injection of ponasterone A (750 μg) were stained for β-galactosidase. Strong induction of β-galactosidase is seen in the mammary gland (*B*), but no significant staining was observed in other organs except for low level expression in the salivary gland (*C*). The salivary gland expression is consistent with the expression of the MMTV transgene encoding VgEcR in the salivary gland.

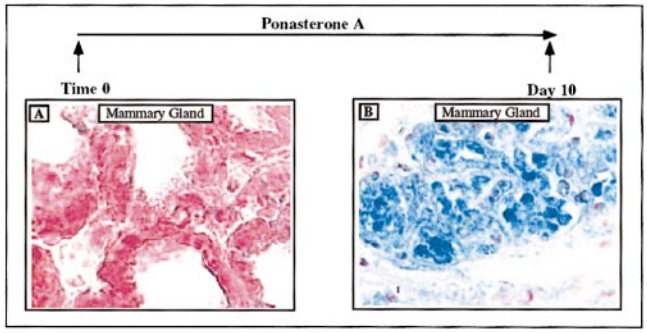


Figure 5. Sustained induction of ecdysone-inducible mammary gland transgene *in vivo*. Ponasterone A pellets (200 μg) were implanted into the transgenic reporter mice and the mammary gland was analyzed for β-galactosidase staining (*A)* preimplantation. *B*) 10 days after implantation, β-galactosidase staining is observed in the mammary epithelial cells. Costaining was performed with fast red.

DISCUSSION

Several types of conditional signaling systems have been devised to regulate genetic or physiological responses with exogenously administered chemicals. Each system conveys unique advantages for specific types of analysis. Conditional systems designed to initiate protein-protein dimerization that reconstitute activity of intracellular signaling pathways use compounds such as rapamycin, coumermycin, or FK1012 (23–25). Alternatively, chimeric transcription factors or mutant receptors can be regulated by tetracycline, mifepristone, tamoxifen, or synthetic small molecules (4, 6, 26). Transgenic systems for functional analysis of genes involved in mammary gland development and disease, particularly tumorigenesis, should ideally combine features of low basal level activity, robust induction by a ligand that is rapidly cleared from the serum, and have homogeneous transgene expression in mammary epithelium. The current studies describe the generation of a transgenic mouse model in which spatial- and temporal-specific mammary gland transgene expression regulated by the ecdysteroid ponasterone A combines these features.

A practical limitation in the past for these studies was the ready availability of an ecdysteroid ligand and evidence of its sustained function *in vivo*. In the current studies, serum clearance of ponasterone A

was rapid with an activity half-life of 48 min. We identified a source of bioactive ponasterone A and extracted milligram quantities using simple techniques (17 and data not shown) and demonstrated sustained transgene expression induced by s.c. implants. Transgene expression was induced within 24 h and sustained using the s.c. implantation of ponasterone A pellets. Bioactive ponasterone A was readily measured in the mammary tissue of the transgenic mice implanted with ponasterone A pellets. The triply transgenic mice have both a similar litter size to control litter mates (Tg. 8.0±1.2, n=14 litters vs. Ctrl 9.0±1.5, n=15 litters) and normal breast morphology by mammary gland whole mount (not shown). Other steroid-inducible transgenic systems have also shown promising results. The steroid ligand tamoxifen induced partial reporter gene expression within 24 h in transgenic mice, with evidence of activity sustained for 4 days (8). The intracellular level of tamoxifen appeared to be a limiting factor in the kinetics of inducing transgene expression (8). In cultured cells (27) and in transgenic mice (28), chimeric progesterone receptors were modulated by the anti-progesterone RU486. Although concerns exist that the concentrations of RU486 required to regulate the chimera in transgenic mice antagonize endogenous progesterone and glucocorticoid receptors, the short half-life of RU486 suggests that the side effects would be transient, implying promise for this system's applicability to transgenics. The steroid ligands RU486 and tamoxifen can affect endogenous mammary gland nuclear receptors, breast epithelial cell cycle progression, and cellular proliferation (29), limiting their potential applicability for analysis of mammary gland gene function. In contrast, ponasterone A did not affect the cell cycle in MCF7 cells at concentrations that induced reporter activity by 50-fold (data not shown), implying that ponasterone A may be an ideal ligand for the analysis of genes involved in cell cycle regulation.

In the current studies, transgene expression within the mammary epithelium of the receptor/enhancer transgenic mice was homogeneous. Alternative mammary gland-directed promoters, including the *whey acidic protein* gene, resulted in mosaic transgene expression (30); its activity during puberty and pregnancy cannot be controlled experimentally (31). Our findings of homogeneous transgene expression contrast with results using a different MMTV-LTR in which mosaic expression and heterogeneous staining of β-galactosidase presented practical limitations for analysis of mammary gland-specific gene function (18). The predominant expression of the inducible transgene in mammary tissue we observed also contrasts with previous studies in which ectopic expression of the tet-regulated expression system was observed (18). We did not observe ectopic transgene expression in the heart, lung, or spleen after the addition of ponasterone A. In contrast, ectopic basal and tet-induced transgene expression was observed in these same tissues of MMTV-LTR-driven, tet-regulated transgenic mice (18). It is likely that the improved expression profile and tissue specificity we observed was due to the type of MMTV promoter (14) driving the expression of the VgEcR.

The enhancer/receptor transgenic mice described here have broad applicability for genetic analysis of mammary gland development and disease states. The low basal level and rapid induction of transgene expression observed with the ecdysone system may be advantageous in regulating CRE-mediated expression and somatic excision. The low basal level expression of transgenic mice expressing tamoxifen-regulated CRE expression was associated with successful ligand-inducible excision of a floxed CAT cassette targeted to the skin (8) as well as CRE-mediated recombination targeted to the neural tube during development (9). As the ecdysone system has been used to induce FLP recombinase in cultured cells (32), the current studies suggest that ponasterone A-induced, mammary gland-specific CRE expression could be used for mammary gland genetic analysis *in vivo*. This transgenic model may be used to analyze the function of putative mammary gland tumor suppressors when mated with mammary gland oncogenic mouse models, for analysis of functional interaction between cooperating oncogenes, and for analysis of dominantly acting oncogenes induced postnatally (33, 34). FJ

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REFERENCES

1. Rossant, J., and McMahon, A. (1999) 'Cre'-ating mouse mutants—a meeting review on conditional mouse genetics. *Genes Dev.* **13,** 142–145
2. Byrne, G. W., and Ruddle, F. H. (1989) Multiplex gene regulation: a two-tiered approach to transgene regulation in transgenic mice. *Proc. Natl. Acad. Sci. USA* **86,** 5473–5477
3. Rundle, C. H., Macias, M. P., Yueh, Y. G., Gardner, D. P., and Kappen, C. (1998) Transactivation of Hox gene expression in a VP16-dependent binary transgenic mouse system. *Biochim. Biophys Acta* **1398,** 164–78
4. Gossen, M., Freunlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Transcriptional activation of tetracycline in mammalian cells. *Science* **268,** 1766–1769

5. Furth, P. A., Onge, L. S., Boger, H., Gruss, P., Gossen, Kistner, A., Bujard, H., and Hennighausen, L. (1994) Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc. Natl. Acad. Sci. USA* **91,** 9302–9306
6. Redfern, C. H., Coward, P., Degtyarev, M. Y., Lee, E. K., Kwa, A. T., Hennighausen, L., Bujard, H., Fishman, G. I., and Conklin, B. R. (1999) Conditional expression and signaling of a specifically designed Gi-coupled receptor in transgenic mice. *Nat. Biotechnol.* **17,** 165–169
7. Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., and Chambon, P. (1996) Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci. USA* **93,** 10887–90
8. Brocard, J., Warot, X., Wendling, O., Messaddeq, N., Vonesch, J. L., Chambon, P., and Metzger, D. (1997) Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc. Natl. Acad. Sci. USA* **94,** 14559–63
9. Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K., and McMahon, A. P. (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8,** 1323–1326
10. Yao, T. P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P., and Evans, R. M. (1993) Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature (London)* **366,** 476–479
11. Yao, T. P., Segraves, W. A., Oro, A. E., McKeown, M., and Evans, R. M. (1992) Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* **71,** 63–72
12. No, D., Yao, T.-P., and Evans, R. M. (1996) Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA* **93,** 3346–3351
13. Watanabe, G., Howe, A., Lee, R.J., Albanese, C., Shu, I.-W., Karnezis, A. N., Zon, L., Kyriakis, J., Rundell, K., and Pestell, R. G. (1996) Induction of cyclin D1 by simian virus 40 small tumor antigen. *Proc. Natl. Acad. Sci. USA* **93,** 12861–12866
14. Guy, C. T., Cardiff, R. D., and Muller, W. J. (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic model for metastatic disease. *Mol. Cell. Biol.* **12,** 954–961
15. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. (1988) Single step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. *Cell* **54,** 105–115
16. Fire, A., Harrison, S. W., and Dixon, D. (1990) A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93,** 189–198
17. Imai, S., and Fujioka, S. (1967) Extraction of ponasterone A and ecdysterone from podocarpaceae and related plants. *Steroids* **10,** 557–565
18. Hennighausen, L., Wall, R. J., Tillmann, U., Li, M., and Furth, P. A. (1995) Conditional Gene expression in secretory tissues and skin of transgenic mice using the MMTV-LTR and the tetracycline responsive system. *J. Cell. Biochem.* **59,** 463–472
19. Cherbas, P., Trainor, D. A., Stonard, R. J., and Nakanishi, K. (1982) 14-Deoxymuristerone, a compound exhibiting exceptional moulting hormonal activity. *J. Chem. Soc. Chem. Commun.* 1307–1308
20. Kalant, H., and Roschau, W.H.E., eds (1998) Pharmacokinetics. In *Principles of Medical Pharmacology*, 6th Ed, pp. 55–66, Oxford University Press, New York
21. Cohen, P. E., and Milligan, S. R. (1993) Silastic implants for delivery of oestradiol to mice. *J. Reprod. Fertil.* **99,** 219–223
22. Muller, W. J., Arteaga, C. L., Muthuswamy, S. K., Siegel, P. M., Webster, M. A., Cardiff, R. D., Meise, K. S., Li, F., Halter, S. A., and Coffey, R. J. (1996) Synergistic interaction of the Neu proto-oncogene product and transforming growth factor alpha in the mammary epithelium of transgenic mice. *Mol. Cell. Biol.* **16,** 5726–5736
23. Ho, S. N., Biggar, S. R., Spencer, D. M., Schreiber, S. L., and Crabtree, G. R. (1996) Dimeric ligands define a role for transcriptional activation domains in reinitiation. *Nature (London)* **382,** 822–6
24. Farrar, M. A., Alberol-Ila, J., and Perlmutter, R. M. (1996) Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. *Nature (London)* **383,** 178–181
25. Spencer, D. M., Wandless, T. J., Schreiber, S. L., and Crabtree, G. R. (1993) Controlling signal transduction with synthetic ligands. *Science* **262,** 1019–1024
26. Wang, Y., O'Malley, B. W. J., Tsai, S. Y., and O'Malley, B. W. (1994) A regulatory system for use in gene transfer. *Proc. Natl. Acad. Sci. USA* **91,** 8180–8184
27. Wang, Y., Xu, J., Pierson, T., O'Malley, B. W., and Tsai, S. Y. (1997) Positive and negative regulation of gene expression in eukaryotic cells with an inducible transcriptional regulator. *Gene Ther.* **4,** 432–441
28. Kellendonk, C., Troche, F., Casanova, E., Anlag, K., Opherk, C., and Schutz, G. (1999) Inducible site-specific recombination in the brain. *J. Mol. Biol.* **285,** 175–182
29. Pestell, R. G., Albanese, C., Reutens, A. T., Segall, J. E., Lee, R. J., and Arnold, A. (1999) The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. *Endocr. Rev.* **20,** 501–534
30. Li, B., Greenberg, N., Stephens, L. C., Meyn, R., Medina, D., and Rosen, J. M. (1994) Preferential overexpression of a 172Arg–>Leu mutant p53 in the mammary gland of transgenic mice results in altered lobuloalveolar development. *Cell Growth Differ.* **5,** 711–721
31. Burdon, T., Sankaran, L., Wall, R. J., Spencer, M., and Hennighausen, L. (1991) Expression of a whey acidic protein transgene during mammary development. Evidence for different mechanisms of regulation during pregnancy and lactation. *J. Biol. Chem.* **266,** 6909–6914
32. Sawicki, J. A., Monks, B., Morris, R.J. (1998) Cell-specific ecdysone-inducible expression of FLP recombinase in mammalian cells. *BioTechniques* **25,** 868–875
33. Kwan, H., Pecenka, V., Tsukamoto, A., Parslow, T.G., Guzman, R., Lin, T. P., Muller, W. J., Lee, F. S., Leder, P., and Varmus, H. E. (1992) Transgenes expressing the Wnt-1 and int-2 proto-oncogenes cooperate during mammary carcinogenesis in doubly transgenic mice. *Mol. Cell. Biol.* **12,** 147–154
34. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. (1987) Coexpression of MMTV/v-Ha-*ras* and MMTV/c-*myc* genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell* **49,** 465–475

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