PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 9–14

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Evidence for two progesterone receptor binding sites in murine mammary carcinomas

Luisa A. Helguero, Caroline Lamb, Alfredo A. Molinolo, Claudia Lanari*

Laboratory of Hormonal Carcinogenesis, Instituto de Biología y Medicina Experimental, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Vuelta de Obligado 2490, C1428AND Buenos Aires, Argentina

Received 29 August 2002

Abstract

We have evaluated the progesterone receptor (PR) binding patterns in progestin-dependent and -independent murine mammary carcinomas; all variants regress completely after antiprogestin treatment. These studies revealed the presence of a high affinity, low capacity-binding site (K_d: 43 ± 9 pM; Q = 9 ± 3 fmol/mg protein) and of the classical lower affinity, high capacity-binding site (K_d: 9.2 ± 4.2 nM; Q = 376 ± 64 fmol/mg protein). These sites could also be detected in uterus. Antiprogestins were able to bind to both sites. In vitro, medroxyprogesterone acetate (MPA) was stimulatory along a biphasic curve with two slopes, one at very low concentrations (EC_{50}: 1.5 ± 0.7 fM) and the other at values compatible with the described K_d for the PR (EC_{50}: 0.33 ± 0.3 nM).

Keywords: Progesterone receptors; Binding sites; Mammary carcinomas; Mice; Hormone dependence; Hormone response; Two sites; Uterus

1. Introduction

In preliminary experiments in mouse mammary carcinomas induced by medroxyprogesterone acetate (MPA), we had observed that the in vitro response to MPA was characterized by a biphasic stimulatory curve with increases in cell proliferation at low concentrations. With this in mind, we decided to reanalyze a group of progestin-dependent and -independent tumors induced by MPA, and maintained by syngeneic passages in BALB/c mice [1–3]. All tumors express both estrogen (ER) and progesterone receptors (PR). The antiprogestins mifepristone and onapristone [4], can induce complete regression in most progestin-dependent and -independent tumors, suggesting that PR are involved in progestin-independent tumor growth [5]. This presumption was also supported by experiments in which PR antisense oligonucleotides also inhibited cell growth [5].

By Scatchard analysis, we identified a previously unreported new high affinity progestin binding site in mouse progestin-dependent and -independent tumor tissue, as well as in uteri. In this paper, we provide experimental evidence for proliferative effects of progestins at concentrations far below the classical K_d for steroid receptors, and correlate these effects with the description of a new high affinity progesterone binding site. Experiments are being carried out to further reveal the nature of these binding sites.

2. Materials and methods

2.1. Animals

Two-month-old BALB/c female virgin mice were used throughout. The animals were fed ad libitum and kept in air-conditioned rooms at 20 ± 2 °C with a 12 h light–dark period. Animal care and manipulation was in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [6].

2.2. Tumors

MPA-induced mammary ductal carcinomas maintained by in vivo syngeneic transplantation were used in all the experiments [7,8]. These included a progestin-dependent (C4-HD) and three progestin-independent tumors (C7-2-HI, CC4-HI, and 59-2-HI). Fragments of each tumor were transplanted subcutaneously to 2-month-old virgin BALB/c female mice. The progestin-dependent tumor was transplanted simultaneously with MPA (20 mg depot sc) in the contralateral flank, and some animals were left untreated

* Corresponding author. Tel.: +54-11-4783-2869; fax: +54-11-4786-2564.
E-mail address: clanari@dna.uba.ar (C. Lanari).

0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(02)00267-4

as a progestin-dependent growth control. Groups of three animals were used in the in vivo experiments and the tumor growth was monitored every 2 days and tumor size measured (length × width) with a vernier caliper.

2.3. Reagents

MPA depot (Medrosterona) was a gift from Gador Laboratories, Buenos Aires. Trypsin, albumin, collagenase type II were purchased from Gibco BRL, NY. [^{3}H]-R5020, [^{3}H]-thymidine and R5020 were purchased from NEN, Boston, MA. Dithiothreitol, EDTA, MPA, 17-β-estradiol (E_2) and culture media Dulbecco's modified Eagle's medium: Ham's F12 (DMEM/F12, 1:1, without phenol red) were purchased from Sigma, St. Louis, MO. Activated charcoal was obtained from Mallinckrodt Chemical Works, NY. Fetal calf serum (FCS) was purchased from Life Technologies Inc. or Gen S.A., Buenos Aires.

2.4. Primary cultures

Epithelial and fibroblastic cells were separated by a modification of the sedimentation technique previously described [9]. The isolated epithelial cells were plated in culture flasks with DMEM/F12 + 5% steroid stripped FCS (ssFCS) and allowed to attach for 24–48 h. At confluence, the cultures were trypsinized and the cell suspensions were used for [^{3}H]-thymidine uptake assays.

2.5. [^{3}H]-Thymidine uptake assay

About 10^4 cells were seeded per well in a 96-well microplate with DMEM/F12+5% ssFCS and allowed to attach for 24 h. The cells were then incubated for 48 h with the experimental solutions, diluted in DMEM/F12 + 2.5% ssFCS. After 24 h incubation, half of the solution was replaced by fresh one and 0.4 μCi of [^{3}H]-thymidine was added. The next day, the cells were trypsinized, harvested and the filters were counted in a β-counter. The assays were performed in octuplicates. Mean and standard deviation were calculated for each solution tested, and the results expressed as proliferation index (experimental cpm/control cpm).

2.6. Statistical studies

ANOVA followed by Tukey *t*-test was used to analyze the differences between control and experimental groups in [^{3}H]-thymidine uptake assays.

2.7. Tissues and tumors used for PR binding

Uteri from adult mice were treated with E_2 10 μg/kg in daily sc injections for 2 days (uterus + E_2). The tumors were excised when they reached approximately 50–100 mm^2 and were immediately frozen in liquid nitrogen.

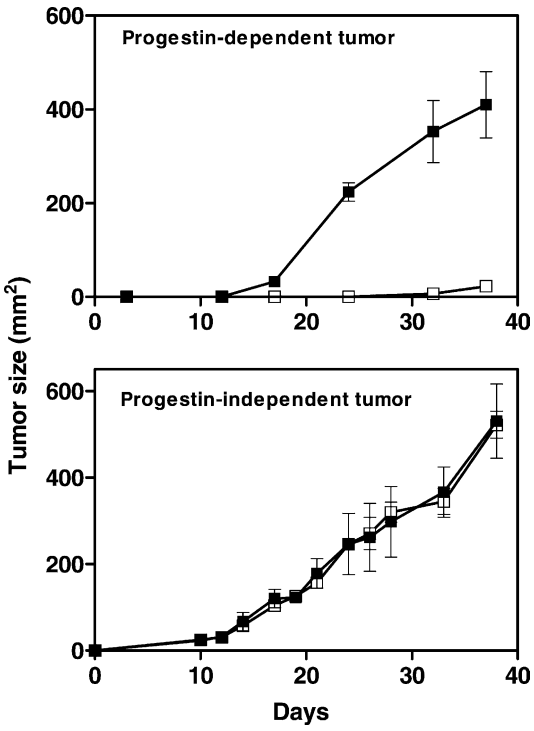

Fig. 1. Representative growth curve of progestin-dependent and -independent tumors. Tumors were inoculated in the right inguinal flank simultaneously with MPA depot (20 mg/kg) in the contralateral flank (■) or left untreated (□).

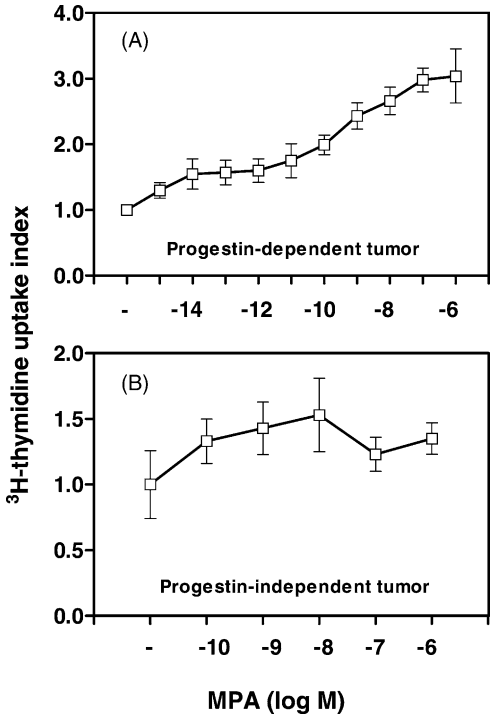

Fig. 2. MPA effect on [^{3}H]-thymidine uptake in primary cultures from progestin-dependent and -independent tumors. (A) $P < 0.001$ from MPA 10^{-14} M; (B) MPA 10^{-10}: $P < 0.05$; MPA 10^{-9} and 10^{-8} M: $P < 0.001$. A representative experiment of other three is shown. [^{3}H]-thymidine index represents the ratio between cpm of experimental group and control group.

2.8. Preparation of total cellular extracts

Tissues and tumors were homogenized in a polytron at setting 50 with three bursts of 5-s in a 1:4 proportion tissue:buffer TEDG. The buffer was 20 mM Tris–HCl pH 7.4, 1.5 mM EDTA, 0.25 mM dithiothreitol, 20 mM Na_2MoO_4, 10% glycerol. The homogenate was centrifuged for 30 min at 12,000 rpm, 4 °C. Protein concentration was determined according to Lowry [10].

2.9. Displacement experiments

PR were labeled by incubating duplicate aliquots of the extracts with 3 nM [^{3}H]-R5020 and displaced with unlabeled R5020, MPA, mifepristone or onapristone in concentrations ranging from 0.5 pM to 20 μM. The reaction proceeded for 18 h at 0 °C. To block undesired binding to serum proteins, a 100-fold excess of unlabeled cortisol was used. To separate free from bound hormones, charcoal–dextran solution was added (buffer TEDG, 1% activated charcoal, 0.1% dextran) and after 10 min, the samples were centrifuged at 3500 rpm, 10 min, at 4 °C. The supernatant was counted in a β-counter. The results were plotted as % bound (bound cpm in each point/total cpm bound) versus log concentration (log nM) and the EC_{50} calculated.

2.10. Saturation experiments

Total cellular extracts were incubated with [^{3}H]-R5020 in concentrations ranging from 3 pM to 30 nM in the presence and absence of 3 μM of unlabeled R5020. To block unspecific binding to serum proteins, glucocorticoid and androgen receptor, 100-fold excess cortisol and 5α-dihydrotestosterone was added to the reaction, which proceeded for 18 h at 4 °C. At the end of the incubation, bound and unbound hormones were separated by charcoal adsorption.

The effective concentration 50 (EC_{50}), dissociation constant (K_d) and total number of receptor sites (Q) were calculated using GraphPad® software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Tumor growth

C4-HD started to grow in MPA-treated animals 10 days after inoculation; only after more than a month was visible growth evident in non-treated animals. For progestin-independent lines, there were no significant growth

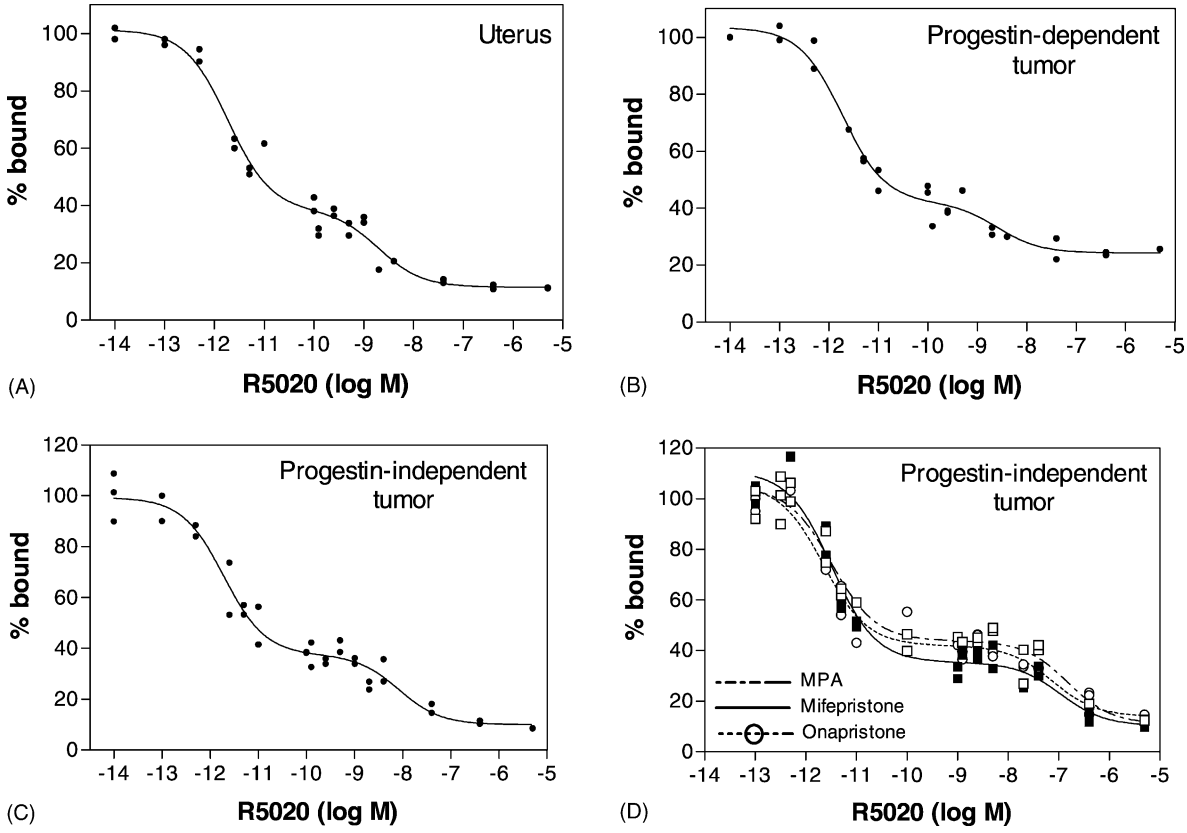

Fig. 3. Displacement experiments. Cell extracts were incubated with 3 nM [^{3}H]-R5020 and the hormone displaced with increasing concentrations of unlabeled R5020 (A–C), MPA, mifepristone or onapristone (D). After 18 h of incubation unbound hormone was removed with activated charcoal. The data was fitted to a two site binding model and EC_{50} values were calculated using GraphPad® software.

differences between treated and untreated animals. Representative growth curves of a progestin-dependent (C4-HD) and a progestin-independent line are shown in Fig. 1.

In in vitro studies the effect of MPA on thymidine uptake was studied in progestin-dependent cultures (C4-HD). MPA was stimulatory along a biphasic curve (Fig. 2A). Two slopes were observed, one at very low concentrations (EC_{50}: 1.5 ± 0.7 fM) and the other one at higher concentrations (EC_{50}: 0.33 ± 0.3 nM). In progestin-independent tumors, MPA induced a subtle stimulation already evident at 0.1 nM ($P < 0.05$) (Fig. 2B).

3.2. Binding analysis: displacement studies and scatchard plots

Tissue extracts were incubated with 3 nM [^{3}H]-R5020 and with increasing concentrations of unlabeled R5020 (Fig. 3A–C). Two binding sites were observed in displacement analysis. An EC_{50} of 2.4 ± 0.5 pM ($n = 8$) was calculated for the high affinity site and an EC_{50} of 30.4 ± 28.3 nM ($n = 8$) for the low affinity site. Two antiprogestins, mifepristone and onapristone, and MPA similarly displaced [^{3}H]-R5020 from both binding sites in progestin-independent tumors (Fig. 3D) with EC_{50} values

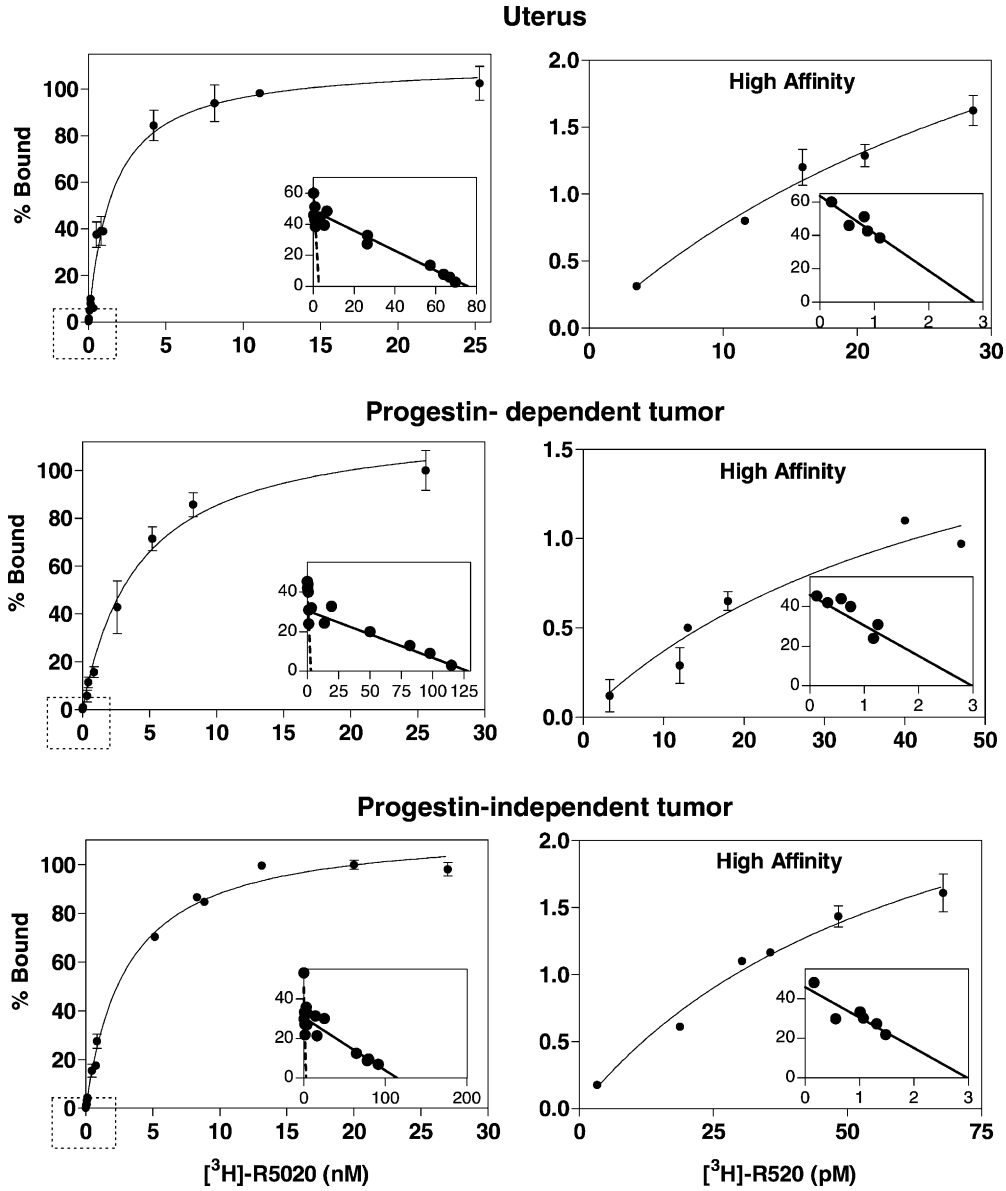

Fig. 4. Saturation experiments and Scatchard analysis (ordinates: bound/free; abscissa: fmol). Cell extracts from normal uteri, a progestin-dependent and -independent tumor were incubated with [^{3}H]-R5020 in concentrations ranging from 5 pM to 30 nM. Left panels: Representative experiments including all points of the curves. Dotted line squares encompassing the lower left vertex of the left panels, corresponding to [^{3}H]-R5020 low range of concentrations, represent the area further analyzed in the right panels. Right panels: in this scale the high affinity site is observed.

of 4.8 ± 1.3 pM and 91.4 ± 9.7 nM for MPA ($n = 2$); 3.1 ± 0.98 pM and 60.24 ± 15.8 nM for mifepristone ($n = 4$) and 4 ± 6.1 pM and 94.1 ± 4.7 nM for onapristone ($n = 2$). Similar results were observed for uteri and progestin-dependent tumors (not shown).

To determine K_d values, we performed conventional saturation assays increasing the number of points within the same range of concentrations used in displacement studies. Only the low affinity sites were detected in the whole analysis (Fig. 4, left panels). The value obtained for uterus, progestin-dependent and -independent tumors was $K_d = 9.2 \pm 4.2$ nM, $Q = 376 \pm 64$ fmol/mg of protein ($n = 13$, pooled values). We then analyzed only the data obtained within the low range of [^{3}H]-R5020 concentrations (0–50 pM), which fit in a saturation curve. K_d and Q for these high affinity, low capacity sites were 43 ± 9 pM, and 9 ± 3 fmol/mg of protein ($n = 6$), respectively (Fig. 4, right panel). The values were similar for uterus, progestin-dependent and -independent tumors. Interestingly, Q values for the low capacity site were always within 2–3% of the total PR content.

4. Discussion

In this study, we have characterized PR binding sites in murine mammary carcinomas with different hormone response. Binding assays revealed the presence of two binding sites, a high affinity and low capacity site (K_d: 43±9 pM) not reported previously, and the classical lower affinity/high capacity site [5]. Both sites were also detected in uterine tissue.

It has long been known that some actions of steroid hormones proceed through mechanisms unrelated to their classical cytosolic/nuclear receptors, and these have collectively been called non-genomic effects [11]. It has been demonstrated for androgens and estrogens in different experimental systems, that very low levels, well under the purported K_d for the classical steroid receptor system, can stimulate cell proliferation [12,13]. Although there is increasing evidence that progesterone can induce proliferation in mammary epithelial cells, not only in animal models [1,14] but also in human breast tissue [15], a similar non-genomic effect for progesterone has not yet been described. We have been able to demonstrate that very low levels (EC_{50}: 1.5 ± 0.7 fM) of progestin induce a reproducible stimulatory effect that can be further increased at higher concentrations (EC_{50}: 0.33 ± 0.3 nM). These results are consistent with two different mechanisms of action, and in coincidence, two binding sites were detected by binding techniques. The high affinity site was hard to visualize because of the relatively low sensitivity of the assay. In fact, we had not been able to detect it in previous studies [5]. MPA, mifepristone and onapristone were able to bind to both sites. In in vitro cell proliferation studies in primary cultures of progestin-independent tumors, MPA was only able to induce a slight increase in [^{3}H]-thymidine uptake. This suggests that the effect regulated by the lower affinity binding site has already been triggered in these tumors. These two sites were also observed in normal uteri. Although the presence of two PR binding sites of 25 and 1 nM has been described in chick oviduct [16], to our knowledge, the high affinity and low capacity-binding sites reported herein, have not yet been described. Further studies are required to elucidate their nature.

Acknowledgements

This work was supported by grants from SECYT: BID 1201/OC-AR, PICT 99 05-06389 and Fundación Sales. We are grateful to Gador Laboratories for kindly providing MPA and to Schering Argentina for providing onapristone. We are also grateful to Dr. I. Lüthy and Dr. J.C. Calvo for orientation with the binding data, to Miss G. Aznarez and J. Bolado for excellent technical assistance in animal care and to Dr C.D. Pasqualini for revising the manuscript.

References

[1] C. Lanari, A.A. Molinolo, C.D. Pasqualini, Induction of mammary adenocarcinomas by medroxyprogesterone acetate in BALB/c female mice, Cancer Lett. 33 (2) (1986) 215–223.
[2] A.A. Molinolo, C. Lanari, E.H. Charreau, N. Sanjuan, C.D. Pasqualini, Mouse mammary tumors induced by medroxyprogesterone acetate: immunohistochemistry and hormonal receptors, J. Natl. Cancer Inst. 79 (6) (1987) 1341–1350.
[3] E. Kordon, C. Lanari, A.A. Molinolo, P.V. Elizalde, E.H. Charreau, C.D. Pasqualini, Estrogen inhibition of MPA-induced mouse mammary tumor transplants, Int. J. Cancer 49 (6) (1991) 900–905.
[4] K.B. Horwitz, L. Tung, G.S. Takimoto, Novel mechanisms of antiprogestin action, Acta Oncol. 35 (2) (1996) 129–140.
[5] M.F. Montecchia, C. Lamb, A.A. Molinolo, I.A. Luthy, P. Pazos, E. Charreau, S. Vanzulli, C. Lanari, Progesterone receptor involvement in independent tumor growth in MPA-induced murine mammary adenocarcinomas, J. Steroid Biochem. Mol. Biol. 68 (1-2) (1999) 11–21.
[6] Institute of Laboratory Animal Resources, Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC, 1996.
[7] C. Lanari, E. Kordon, A. Molinolo, C.D. Pasqualini, E.H. Charreau, Mammary adenocarcinomas induced by medroxyprogesterone acetate: hormone dependence and EGF receptors of BALB/c in vivo sublines, Int. J. Cancer 43 (5) (1989) 845–850.
[8] E.C. Kordon, F. Guerra, A.A. Molinolo, E.H. Charreau, C.D. Pasqualini, P. Pazos, G. Dran, C. Lanari, Effect of sialoadenectomy on medroxyprogesterone-acetate-induced mammary carcinogenesis in BALB/c mice. Correlation between histology and epidermal-growth-factor receptor content, Int. J. Cancer 59 (2) (1994) 196–203.
[9] G. Dran, I.A. Luthy, A.A. Molinolo, E.H. Charreau, C.D. Pasqualini, C. Lanari, Effect of medroxyprogesterone acetate (MPA) and serum factors on cell proliferation in primary cultures of an MPA-induced mammary adenocarcinoma, Breast Cancer Res. Treat. 35 (2) (1995) 173–186.
[10] O.H. Lowry, N.J. Rosebrough, A.L. Farr, Protein measurements with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[11] E.R. Levin, Cellular functions of the plasma membrane estrogen receptor, Trends Endocrinol. Metab. 10 (9) (1999) 374–377.
[12] A. Bandyopadhyay, Y. Zhu, M.L. Cibull, L. Bao, C. Chen, L. Sun, A soluble transforming growth factor beta type III receptor suppresses tumorigenicity and metastasis of human breast cancer MDA-MB-231 cells, Cancer Res. 59 (19) (1999) 5041–5046.
[13] R.X. Song, G. Mor, F. Naftolin, R.A. McPherson, J. Song, Z. Zhang, W. Yue, J. Wang, R.J. Santen, Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17beta-estradiol, J. Natl. Cancer Inst. 93 (22) (2001) 1714–1723.
[14] I.H. Russo, P. Gimotty, M. Dupuis, J. Russo, Effect of medroxyprogesterone acetate on the response of the rat mammary gland to carcinogenesis, Br. J. Cancer 59 (2) (1989) 210–216.
[15] R.K. Ross, A. Paganini-Hill, P.C. Wan, M.C. Pike, Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin, J. Natl. Cancer Inst. 92 (4) (2000) 328–332.
[16] A. Maggi, W.T. Schrader, B.W. O'Malley, Progesterone-binding sites of the chick oviduct receptor. Presence of a weaker ligand site which is destroyed by phosphatase treatment, J Biol. Chem. 259 (17) (1984) 10956–10966.