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PRECLINICAL STUDY

Hypermethylation of the progesterone receptor A in constitutive antiprogestin-resistant mouse mammary carcinomas

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Abstract Most breast carcinomas that are estrogen receptor (ER) and progesterone receptor (PR) positive respond initially to an endocrine therapy, but over time, they develop resistance (acquired hormone resistance). Others, however, fail to respond from the beginning (constitutive resistance). Overcoming hormone resistance is one of the major desirable aims in breast cancer treatment. Using the medroxyprogesterone acetate (MPA)-induced breast cancer mouse model, we have previously demonstrated that antiprogestin-responsive tumors show a higher expression level of PR isoform A (PRA) than PR isoform B (PRB), while tumors with constitutive or acquired resistance show a higher expression level of PRB. The aim of this study was to investigate whether PRA silencing in resistant tumors was due to *PRA* methylation. The CpG islands located in the *PRA* promoter and the first exon were studied by methylation-specific PCR (MSP) in six different tumors: two antiprogestin-responsive, two constitutive-resistant, and two with acquired resistance. Only in constitutive-resistant tumors, PRA expression was silenced by DNA methylation. Next, we evaluated the effect of a demethylating agent, 5-aza-2′-deoxycytidine, on PRA expression and antiprogestin responsiveness. In constitutive-resistant tumors, 5-aza-2′-deoxycytidine treatment in vitro and in vivo restored PRA expression and antiprogestin RU-486 responsiveness. Furthermore, high levels of DNA methyltransferase (Dnmts) 1 and 3b were detected in these tumors. In conclusion, our results suggest that methyltransferase inhibitors in combination with antiprogestins may be effective in the treatment of constitutive-resistant carcinomas with a high DNA methyltransferase level.

Keywords Mammary carcinomas · Hormone resistance · Progesterone receptors · DNA-methylation · DNMT inhibitors

Abbreviations

5azadC	5-Aza-2′-deoxycytidine
Dnmts	DNA methyltransferases
E_2	17-β-Estradiol
ER	Estrogen receptor
ERα	ER alpha
FCS	Fetal calf serum
GR	Glucocorticoid receptor
H&E	Hematoxylin and eosin
HPF	High power field
i.p.	Intraperitoneal
M	Methylated
MPA	Medroxyprogesterone acetate
MSP	Methylation-specific PCR
PI	Propidium iodide
PR	Progesterone receptor
PRA	PR isoform A

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PRB	PR isoform B
RU-486	Mifepristone
s.c.	Subcutaneous
UM	Unmethylated

Introduction

Two-thirds of breast cancers express estrogen receptor (ER) and progesterone receptor (PR) at the time of diagnosis [1]. Most tumors initially respond to endocrine therapy, but many will eventually develop resistance (acquired hormone resistance). However, some tumors fail to respond to endocrine treatment from the beginning (constitutive resistance) despite expressing hormone receptors [2].

Progesterone receptor exists as two isoforms, PRA and PRB, which are transcribed from a single gene under the control of distinct promoters [3, 4]. Both isoforms bind progestins and directly activate the expression of genes that contain progesterone response elements in their promoters. Alternatively, the PRs can cooperate with other transcription factors to induce gene transcription. However, there is increasing evidence that the two isoforms have different functions in vivo [5–8].

We have developed a model of breast cancer in which the administration of medroxyprogesterone acetate (MPA) to female BALB/c mice induces mammary ductal carcinomas [9, 10]. The main features of this tumor model have recently been reviewed [11]. Although these tumors were all originally MPA dependent, some MPA-independent metastatic tumors capable of growing in untreated mice, which retained high levels of ER and PR expression, were obtained by syngeneic transplantation [11]. While most of the MPA-independent tumors regressed in response to antiprogestins (antiprogestin responsive), some of them did not and were designated as constitutive antiprogestin-resistant tumors. From the MPA-independent tumors that regressed with antiprogestins, by selective pressure, we were also able to generate variants with acquired antiprogestin resistance. We have recently reported that PRA is downregulated in both, constitutive and acquired antiprogestin-resistant tumors [12, 13]. Interestingly, the tumors with acquired resistance reverted to the antiprogestin-responsive phenotype following estrogen or tamoxifen treatment or by successive transplantations in untreated mice. Furthermore, in all cases, the reacquisition of antiprogestin responsiveness could be correlated with an increase in PRA expression [13].

It has been shown that one mechanism for the loss of gene expression in oncogenesis is the aberrant methylation of CpG islands in the 5′ regulatory region and first exon of target genes [14]. CpG islands are regions of DNA with several CpG sites, in which a cytosine residue located 5′ of guanine residue is methylated. At least, three types of DNA methyltransferases (Dnmt) exist in mammals: Dnmt1 is a maintenance methylase, while Dnmt3a and Dnmt3b are de novo methylases [15, 16]. Compared to normal cells, cancer cells show a drastic change in DNA methylation, generally exhibiting global DNA hypomethylation as well as region-specific hypermethylation [17]. A correlation between the overexpression of Dnmts and hypermethylation in breast cancer cell lines has been demonstrated [18]. 5-aza-2′-deoxycytidine (5azadC) is a well-known demethylating agent that is activated in vivo and readily incorporated into DNA during replication. As a result of the methyltransferase reaction, the Dnmt becomes covalently linked to DNA, rendering it unable to maintain its methylase activity [15]. Treatment of ERα-negative cells with 5azadC leads to reactivation of functional ERα expression [15, 16, 19].

Methylation of the CpG islands located in the *ER* and *PR* genes has been reported in a significant fraction of ER- and PR-negative primary breast cancers and breast cancer cell lines [20–26]. However, no studies in which the methylation status of steroid receptor genes was evaluated in tumors with acquired resistance have been done. The main goal of our study was to evaluate whether DNA methylation could explain PRA silencing in tumors with acquired or constitutive antiprogestin resistance in our experimental model. We demonstrate that PRA is silenced by DNA methylation in constitutive antiprogestin-resistant carcinomas and, 5azadC treatment restores PRA expression and antiprogestin RU-486 responsiveness in vitro and in vivo. This suggests that different epigenetic mechanisms are involved in constitutive antiprogestin resistance and in acquired antiprogestin resistance. In addition, our studies reinforce a pivotal role for PRA mediating antiprogestin's inhibitory effect, highlighting the differential roles of PRA and PRB.

Materials and methods

Animals

Two-month-old virgin female BALB/c mice (IBYME Animal Facility) were used. Animal care and manipulation protocols were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [27].

Tumors

Mammary carcinomas from the MPA breast cancer model were used: C4-HI, C4-HIR, and C4-2-HI are all MPA-independent variants from the C4 family; 59-2-HI, 59-2-

HIR, and 59-HI are variants from the 59 family of tumors; C4-HI and 59-2-HI are antiprogestin-responsive tumors [13, 28]; C4-HIR and 59-2-HIR are their respective antiprogestin-resistant variants [13]; and C4-2-HI and 59-HI are constitutive-resistant variants [12]. Tumors were named before learning their antiprogestin responsiveness. All these tumor variants express ER and PR evaluated by binding, western blotting, and immunohistochemistry assays [11]. However, low levels of PRA were detected in antiprogestin-resistant tumors [12, 13]. Tumors were transplanted by subcutaneous (s.c.) injection into the inguinal flank of BALB/c mice.

Reagents

The 5azadC, MPA, and RU-486 (mifepristone) were purchased from Sigma-Aldrich (St. Louis, MI), and ZK 230211 was a kind gift from Bayer Schering Pharma AG, Berlin.

Methylation-specific PCR (MSP)

Genomic DNA was extracted from tumors, and 1 μg DNA was subjected to sodium bisulfite conversion as described by Frommer et al. [29] with brief modifications. Sodium bisulfite-modified DNA (150 ng) was used as the template in each PCR reaction. The PCR mixture contained PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer, and 1 unit Platinum *Taq* Polymerase (Invitrogen, Carlsbad, CA). In order to amplify the unmethylated (UM) and methylated (M) CpG sites, the primers listed in Table 1 were used. These primers were designed using Methyl Primer Express Software 1.0 from Applied Biosystems (Foster City, CA) and the sequence of the promoter and the first exon of *PR* (http://www.ensembl.org/Mus_musculus/index.html) [30]. The PCR conditions and the annealing temperatures are indicated in Table 1. In order to obtain DNA from normal lymphocytes to be used in control experiments, axillary and inguinal lymph nodes from 6 BALB/c mice were excised and processed as the tumor samples. The lymphocyte DNA was treated with sodium bisulfite (UM control) or was hypermethylated with *Sss*I methyltransferase (New England Biolabs, Beverly, MA), and subsequently treated with sodium bisulfite (M control). PCR products were visualized in a 2% agarose gel.

Cloning and sequencing of MSP products

The PCR products (UM and M) obtained using the PR4 primer pair from three responsive (C4-HI, 59-2-HI), three constitutive (C4-2-HI, 59-HI), and three acquired antiprogestin-resistant (C4-HIR, 59-2-HIR) tumors from each tumor family were cloned into pCR2.1-TOPO (Invitrogen). This PR4 product was chosen because it had the highest levels of CpG islands. The plasmids were transformed into chemically competent *E. coli* TOP10 (Invitrogen). The bacteria were plated on LB agar plates containing 100 μg/ml of ampicillin and 40 μl/plate of 40 mg/ml X-gal (Promega,

Table 1 Sequence of primers selected for PCR methylation studies with their annealing temperatures and PCR conditions

Primers	Sequence	Annealing *T* (°C)
PR1 F M	5′ GGGCGGGTTTTTTTAGAGC 3′	57
PR1 R M	5′ CTCGTTCTCCTACAACGACA 3′	58
PR1 F UM	5′ TTTTGGGTGGGTTTTTTTAGAGT 3′	58
PR1 R UM	5′ TACTCATTCTCCTACAACAACAA 3′	58
PR2 F M	5′ ATTTTATCGTTATCGGGATAGCGC 3′	62
PR2 R M	5′ ATAAATATAAAATCGCAAAACCCG 3′	57
PR2 F UM	5′ TATTTTATTGTTATTGGGATAGTGT 3′	56
PR2 R UM	5′ AATAAATATAAAATCACAAAACCCA 3′	54
PR3 F M	5′ GAAGAAATACGAAAAAAAGTTTTTC 3′	56
PR3 R M	5′ ATAAATATAAAATCGCAAAACCCG 3′	57
PR3 F UM	5′ AGAAGAAATATGAAAAAAAGTTTTTT 3′	54
PR3 R UM	5′ AATAAATATAAAATCACAAAACCCA 3′	54
PR4 F M	5′ GTTTTTTATACGTTTGGCGTTTC 3′	58
PR4 R M	5′ CACGTCGAACAACGACTACT 3′	58
PR4 F UM	5′ AGGTTTTTTATATGTTTGGTGTTTT 3′	56
PR4 R UM	5′ CTCCACATCAAACAACAACTACT 3′	59

Denaturalization temperature and duration		Annealing *T* (°C)	Extension temperature and duration	Final extension
94°C–5 min	94°C–35 cycles of 45 s	45 s	72°C–45 s	72°C–10 min

Madison, WI). Ten white colonies from each group were analyzed by colony PCR using PR4 primer pair to confirm their positivity. The fragments were sequenced by Macrogen Inc. (Korea) using the 3730XL DNA Sequencer. Sequences were finally analyzed using EMBLE-EBI software (http://www.ebi.ac.uk). The percentage of methylation was calculated for each of the three samples from each tumor, and the mean ± SEM was calculated for each tumor.

Primary cultures and co-cultures

Culture media

DMEM/F12 (Dulbecco's modified Eagle's medium: Ham's F12, 1:1, without phenol red, Sigma Chem. Co. St Louis MO, USA); 100 U/ml penicillin; and 100 μg/ml streptomycin with 10% fetal calf serum (FCS; Life Technologies Inc., Gaithersburg, MD, USA). Steroid-stripped FCS was prepared as described previously [31], and it was used in proliferation assays to avoid the interference with endogenous hormones.

Primary cultures

Epithelial cells and carcinoma-associated fibroblasts were separated by differential sedimentation [32] and plated with 10% FCS. Carcinoma-associated fibroblasts were allowed to attach for 0.5 h and the epithelial cells for 24–48 h. The medium was replaced by fresh medium with 10% FCS; thereafter, it was changed every 2–3 days.

Co-cultures

Trypsinized cells were resuspended and equal amounts of epithelial cells and carcinoma-associated fibroblasts were seeded with 10% FCS that was replaced after attachment by 1% steroid-stripped FCS. As previously demonstrated in these experimental conditions, both cell types are in quiescence, and they only grow in co-cultures [32]. The same amount of epithelial cells or carcinoma-associated fibroblasts or double the amount of purified cells was used for comparison.

Cell proliferation

(^{3}H)-thymidine-uptake was used as an indirect method to evaluate cell proliferation [32]. In brief, cells were seeded into 96-well microplates. After attachment (24 h), the cells were incubated for 24 h with 1% steroid-stripped FCS and then for 48 h with the experimental solutions to be tested in 1% chFCS. Fifty percent of the medium was replaced with fresh medium every 24 h. The cells were incubated with 0.4 μCi of ^{3}H-thymidine (specific activity: 20 Ci/mmol) for 24 h, trypsinized, and harvested in a cell harvester. In experiments in which 5azadC was used, the experiments were carried out using proliferating cells (presence of 10% FCS) to guarantee the effect of the demethylating agent. Three different experiments were made using octuplicates, and the means and standard deviations of one representative experiment of the other three are shown. The results are expressed as the proliferation index (cpm experimental group/cpm control; mean ± SEM) [33].

Treatment of epithelial cells with 5azadC in vitro

C4-2-HI and 59-HI epithelial cells from primary cell cultures were allowed to attach for 24 h. The medium was replaced with fresh medium with 10% FCS, and 5azadC was added for 96 h. The medium was refreshed daily. Cells were processed for western blot or immunofluorescence to evaluate PR expression.

In vivo treatment with RU-486 and 5azadC

C4-2-HI, 59-HI, and C4-HIR tumors were transplanted s.c nearby the mammary gland 4th in syngenic mice, and measured every 2 days (length and width). Treatments were initiated when the tumors were palpable. The antiprogestin RU-486 (mifepristone) was inoculated s.c at a dose of 12 mg/kg/day. The mice were inoculated intraperitoneally (i.p.) with 0.75 mg/kg 5azadC (for C4-2-HI and C4-HIR tumors) or 1 mg/kg 5azadC (for 59-HI tumors) every other day. All the experiments were repeated twice using five mice per group. The animals were euthanized after 13 days of treatment, and tumor samples were removed and frozen at −80°C or fixed in 10% formaldehyde.

Western blots

Cytosolic or nuclear extracts were processed for western blots as described previously [12]. The cells were lysed using Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) according the manufacturer's instructions. The western blot membranes were incubated with antibodies against PR (C-19, Santa Cruz Biotech, CA, or Ab-7, Neomarkers, Lab Vision Corp, Fremont, CA), PRB (Ab-6, Neomarkers), ERKs (SC-94, Santa Cruz Biotech), E-Cad (610182, BD), Actin (I-19, Santa Cruz Biotech), Dnmt1, Dnmt3a and Dnmt3b (H-300, H-295, and H-230, respectively, Santa Cruz Biotech), RARβ (SC-14028, Santa Cruz Biotech), PTEN (Ab32199, Abcam), p16 (SC-1207, Santa Cruz Biotech), Rb (SC-50, Santa Cruz Biotech), or glucocorticoid receptors (GRs) (SC-1004, Santa Cruz Biotech) overnight at 4°C, at a concentration of 2 μg/ml in PBST (0.8% NaCl, 0.02% KCl, 0.144%

Na_2PO_4, 0.024% KH_2PO_4, pH 7.4, 0.1% Tween 20). The band intensities from 3 to 4 different tumor samples in different western blots were quantified using Image Quant software.

Immunofluorescence

Frozen sections or cells grown in chamber slides, fixed in 70% ethanol for 1 h, were incubated with antibodies recognizing Dnmt1, Dnmt3a, Dnmt3b, PRA (Ab-7), RPB (Ab-6), or GR, in blocking buffer at a 1:200 dilution overnight at 4°C. They were then incubated with a FITC-conjugated anti-rabbit (FI-1000, Vector Laboratories Burlingame, CA; 1:100 dilution) secondary antibody for 1 h at room temperature. Nuclei were stained with propidium iodide (PI, Sigma). The slides were mounted using Vectashield (Vector Laboratories). Stained cells were analyzed using a Nikon Eclipse E800 Laser Confocal Microscope and EZ-C1 2.20 software. Cell staining was quantified using Image Quant software.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tissue were processed as previously described [28] and stained with the PRA-specific antibody (C-19) using the avidin–biotin–peroxidase complex technique (Vectastain Elite ABC kit; Vector), as described previously [28].

Morphological studies

H&E stained sections of livers, spleens, and kidneys from the 5azadC-treated mice (1 mg/kg or 0.75 mg/kg/every other day; $n = 5$) were studied by an expert mouse pathologist. The percentage of tumor stroma versus tumor parenchyma was quantified with the ImageJ software in treated and untreated tumors. The stromal area in relation to the total tumor area was calculated in five representative fields of each sample, using 400× magnification, in three different tumor samples of each group, and the mean ± SEM was calculated. Mitotic and apoptotic indices were counted in 10 and 15 high-power fields (HPFs), respectively, of each section, using 1000× magnification, and expressed as the mean ± SEM of the percentage of the ratios between the total number of events (mitosis or apoptosis) and the total cell number per HPF. Mitotic figures were identified morphologically by the condensed ''hairy'' aspect of the chromosomes. Morphological identification of apoptosis was performed according to criteria previously reported, which correlated with the deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method [28].

Statistical analysis

Data were analyzed using ANOVA and the Tukey multiple post t test (for multiple samples) or the Student's t test to compare the mean ± SD using Graph Prism 4.0 software. Tumor growth curves were studied using regression analysis and slopes compared using analysis of variance followed by parallelism analysis.

Results

Carcinomas with acquired resistance regain their hormone responsiveness and the PRA/PRB ratio when cultured on plastic

We have previously reported on several MPA-independent variants that were generated from MPA-induced mammary carcinomas. The two tumor families used herein are depicted in Fig. 1. C4-HD tumor gave rise to C4-HI, which responds to antiprogestin treatment, and to C4-2-HI, which showed constitutive hormone resistance. Similarly, 59-HD gave rise to 59-HI, a constitutive-resistant tumor, and to 59-2-HI, an antiprogestin-responsive variant [8]. From both of the antiprogestin-responsive tumors, we developed variants with acquired resistance (C4-HIR and 59-2-HIR) by selective pressure using RU-486 [13] (Figs. 1, 2a). We have recently reported an inverse PRA/PRB ratio in responsive tumors (C4-HI, 59-2-HI) as compared with the resistant variants, with PRA being higher than PRB [13].

We were interested in evaluating whether carcinoma-associated fibroblasts contributed to the resistant phenotype; so, we first evaluated hormone responsiveness in purified epithelial cells from the three different tumor types. As expected, MPA induced a strong proliferative effect in C4-HI cells ($P < 0.001$), which was abolished by RU-486 or ZK 230211 ($P < 0.001$), whereas no differences were observed in C4-2-HI cells. However, unexpectedly, a proliferative effect was observed in the acquired resistant C4-HIR cells treated with MPA ($P < 0.001$), which was abolished by RU-486 or ZK 230211 ($P < 0.001$; Fig. 2a), indicating that acquired antiprogestin resistance but not constitutive resistance is reversed by in vitro culturing. Similar results were obtained with the 59 family of tumors (not shown).

The expression level of PRA and PRB in purified tumor cells from the three tumors growing on plastic was analyzed using western blot (Fig. 2b) and immunofluorescence (Fig. 2c). The PR isoform ratio in C4-HIR cells became similar to that of the responsive tumors (Fig. 2b, c), whereas the PR isoform pattern did not change in the constitutive antiprogestin-resistant tumors (Fig. 2c). These results suggest that different mechanisms regulate PRA

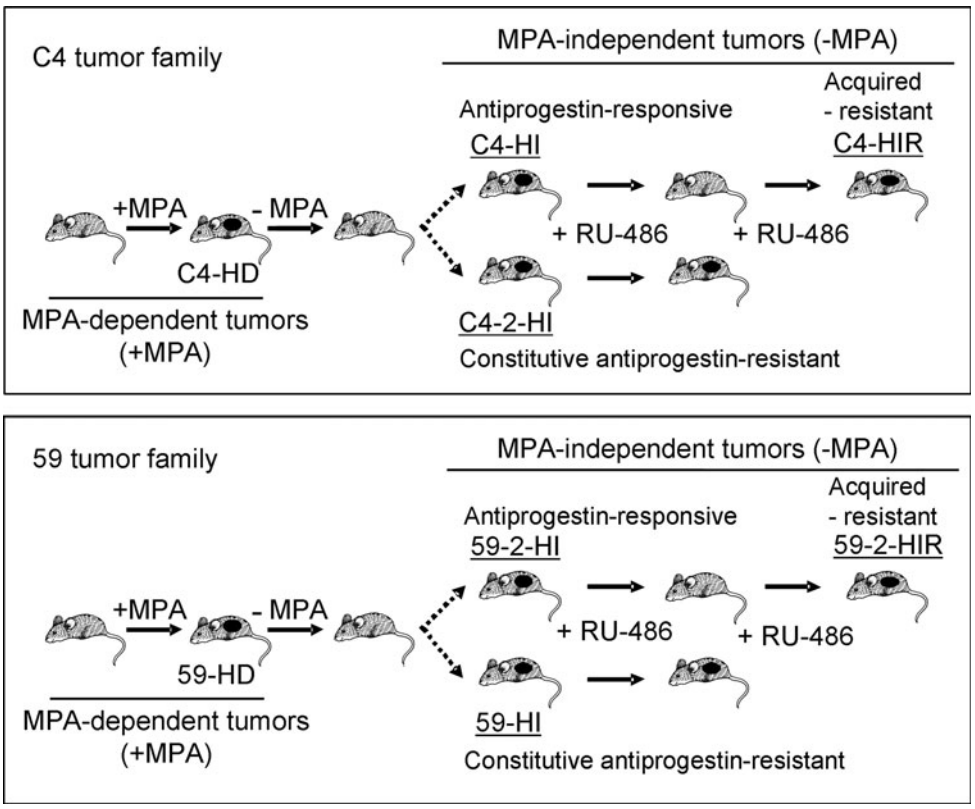

Fig. 1 Origin of the tumors of the MPA breast cancer model used in this study. MPA-induced ductal hormone-dependent mammary carcinomas are maintained by syngeneic transplantation in progestin-treated BALB/c mice (C4-HD and 59-HD). Occasionally, some tumors started to grow in untreated mice giving rise to MPA-independent variants. These variants were named chronologically prior to testing their hormone responsiveness. While most of the MPA-independent tumors regressed in response to RU-486 (C4-HI and 59-2-HI), some constitutive-resistant variants were obtained (C4-2-HI and 59-HI). In addition, MPA-independent responsive tumors treated with RU-486 gave rise, by selective pressure, to acquired antiprogestin-resistant variants (C4-HIR and 59-2-HIR). Tumors used in this study are *underlined*

silencing in tumors with constitutive and acquired antiprogestin resistance.

The incubation of epithelial tumor cells with carcinoma-associated fibroblasts does not modify their hormone responsiveness

In order to investigate whether carcinoma-associated fibroblasts could be participating in the acquired antiprogestin-resistant phenotype, purified epithelial cells from the antiprogestin-responsive tumor C4-HI or from the acquired antiprogestin-resistant tumor C4-HIR were co-cultured with equal amounts of their own carcinoma-associated fibroblasts or with those from the other tumor, as described previously [32]. In both cases, MPA stimulated, and RU-486 inhibited co-culture cell proliferation (Suppl. Fig. 1), suggesting that the presence of carcinoma-associated fibroblasts does not change the hormone responsiveness.

PRA expression is silenced by methylation only in constitutive antiprogestin-resistant tumors

In order to investigate the mechanisms involved in PRA silencing, we carried out methylation analyses of the *PRA* promoter using the same tumors studied above. Different CpG sites were analyzed with four different pairs of primers (Table 1). Figure 3a shows a schematic of the *PR* promoter, with the locations of the CpG sites of both PR isoforms. As expected, only unmethylated CpG islands were observed in the two antiprogestin-responsive tumors (C4-HI and 59-2-HI; Fig. 3b). Hypermethylation of the *PRA* promoter was detected in the two constitutive antiprogestin-resistant tumors (C4-2-HI and 59-HI), whereas the *PRA* promoter in both tumors with acquired antiprogestin resistance (C4-HIR and 59-2-HIR) was unmethylated (Fig. 3b). Although we observe some methylated CpG islands using the PR1 pair of primers that are included in the PRB promoter, the density is not enough for these islands to qualify for a mechanism of gene silencing by promoter methylation (http://www.ensembl.org/Mus_musculus/index.html; [30]). In these experiments, DNA from normal lymphocytes treated with *Sss*I methyltransferase was used as the methylated control, while untreated DNA was included as an unmethylated control (Fig. 3c).

The PCR products from the reactions using the PR4 primer pair and DNA from the antiprogestin-responsive tumors and the tumors with acquired and constitutive antiprogestin resistance were cloned and sequenced to analyze the degree of CpG island methylation. Whereas 100% of the CpG sites were unmethylated in the responsive and acquired resistant tumors (C4-HI, 59-2-HI, C4-HIR and 59-2-HIR), 89.9 ± 2.2% of the CpG sites were methylated in C4-2-HI, and

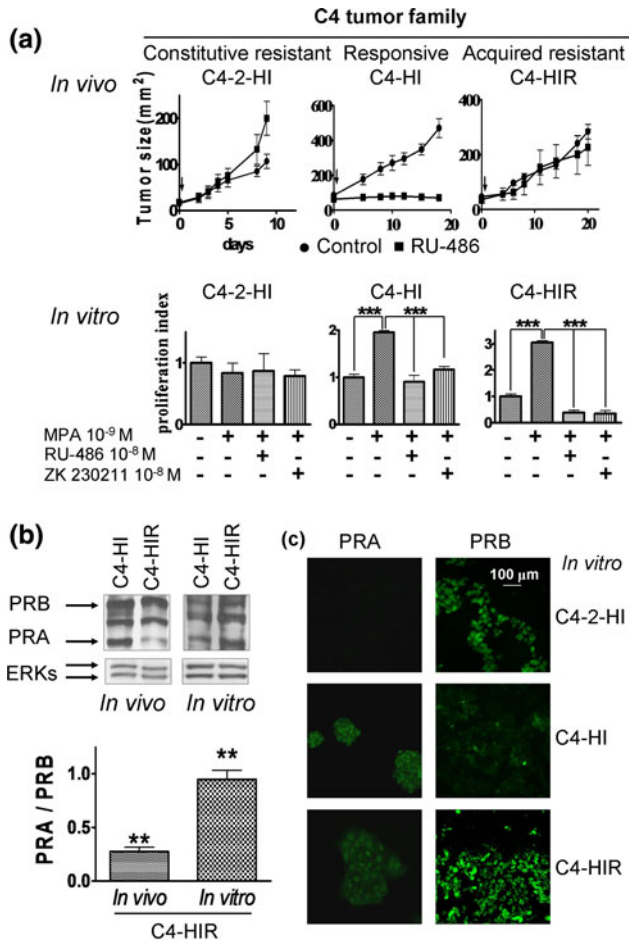

Fig. 2 Acquired antiprogestin-resistant tumors revert their antiprogestin resistance and the PRA/PRB ratio in culture. **a** Hormone responsiveness. *Top* Growth curves from tumors of the C4 family illustrating their hormone responsiveness (already published); *bottom* Primary cultures of purified epithelial cells from the same tumors were subcultured in 96-well microplates. After attachment, the medium was replaced by 1% chFCS. The cells were then treated for 48 h with MPA with or without RU-486 or ZK 230211. Proliferation index was calculated as experimental cpm/control cpm (mean ± SEM), and a representative experiment from the three, using octuplicates in each experiment, is shown. C4-HIR tumors acquired MPA and antiprogestin responsiveness when cultured in vitro; *** $P < 0.001$. **b** Western blots. Representative blots for PRA (83 kDa) and PRB (115 kDa) using nuclear extracts from the tumors or cultures. The polyclonal rabbit C-19 antibody was used. ERKs were used as loading controls. The ratio of PRA/PRB in three different blots using different samples was quantified. C4-HIR tumors cultured on plastic reverted the PRA/PRB ratio ($P < 0.01$; bottom). **c** Immunofluorescence for PRA (Ab-7) and PRB (Ab-6) in cells growing on plastic. Cells were seeded in chambers slides, starved for 24 h, then fixed, and incubated with Ab-7 or Ab-6 antibodies as described in "Materials and methods" section. FITC-conjugated mouse secondary antibodies were used. No staining was observed in the absence of the primary antibodies (not shown). Cells with acquired resistance re-expressed PRA: bar: 100 µm

88.8 ± 3.2% of the CpG sites in 59-HI constitutive-resistant tumors. A diagram illustrating the CpG sites that were methylated in one representative sample is shown in Fig. 3d.

We conclude that methylation of CpG sites at the *PRA* promoter explains PRA silencing only in the constitutive antiprogestin-resistant tumors.

In vitro treatment with 5azadC induces PRA expression and RU-486 responsiveness in constitutive-resistant purified epithelial cells

The strong correlation between PRA expression and the antiprogestin RU-486 responsiveness suggested that constitutive-resistant tumors might be re-sensitized to RU-486 treatment after the restoration of PRA expression. Therefore, we treated these tumors with a demethylating agent, and evaluated their PRA expression and antiprogestin responsiveness.

Primary cell cultures from the two constitutive-resistant tumors, C4-2-HI and 59-HI, were treated with 5azadC (5×10^{-7} and 5×10^{-6} M, respectively) for 96 h. An increase in PRA expression ($P < 0.001$) was observed using immunofluorescence and western blot in both tumor cells (Fig. 4a, b). However, no significant changes were observed using the Ab-6 antibody, which only stains PRB (Fig. 4a).

In order to further investigate whether the restoration of PRA expression by 5azadC treatment could induce antiprogestin responsiveness, primary cultures of C4-2-HI and 59-HI cells were treated with 5azadC plus RU-486, and cell proliferation was evaluated using (^{3}H)-thymidine incorporation. As observed in Fig. 4c, the proliferation of cells treated with 5 µM 5azadC and 100 nM RU-486 was inhibited ($P < 0.001$), whereas treatment with 5azadC or RU-486 alone did not alter cell proliferation.

In vivo 5azadC treatment induces PRA expression and RU-486 responsiveness in constitutive antiprogestin-resistant tumors

In order to investigate whether this restoration of PRA expression and RU-486 responsiveness could be achieved in vivo, mice carrying palpable constitutive-resistant tumors, C4-2-HI and 59-HI, were treated with vehicle, 5azadC, RU-486, or 5azadC in combination with RU-486. Interestingly, as observed in Fig. 5a, the combination of 5azadC and RU-486 significantly inhibited tumor growth in both cases. Morphological signs of tumor regression, including increased stromal tissue intermingled with the epithelial nests ($P < 0{,}001$), increased apoptosis, and a decreased mitotic index, were observed in tumors treated with the combination of RU-486 and 5azadC (Fig. 5b; Table 2). However, no significant differences were observed in tumors treated with RU-486 or 5azadC alone.

In addition, an increase in nuclear and cytoplasmic PRA staining was observed in 5azadC-treated C4-2-HI tumors compared with control tumors (Fig. 5c). Nuclear staining

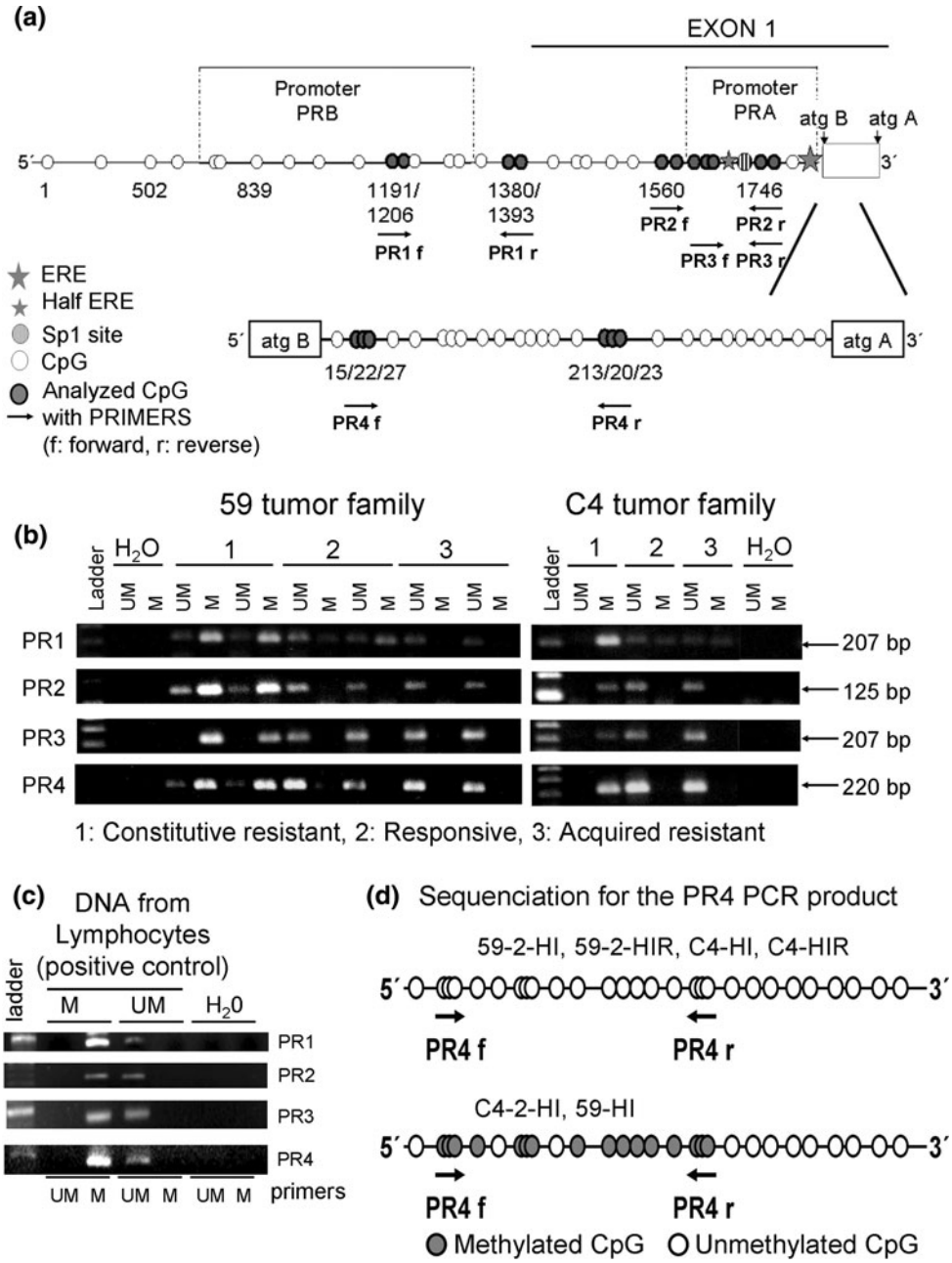

Fig. 3 Methylation studies. **a** Diagram showing the PR promoters and the primers used. ERE: estrogen response element; Sp1: stimulatory protein one. **b** DNA methylation of the PR promoter detected using (MSP). Bisulfite-treated DNA samples from responsive tumors (C4-HI and 59-2-HI), tumors with acquired resistance (C4-HIR and 59-2-HIR) and constitutive-resistant tumors (C4-2-HI and 59-HI) were used for amplification using the specific primers shown in Table 1 and Fig. 3a. Controls without DNA (H_2O) were included. Only tumor samples from constitutive-resistant tumors showed bands using the methylated (M) primers. **c** Normal lymphocytes DNA was used as unmethylated (UM) control, and treated with *Sss*I methyltransferase as M control. **d** The PCR products amplified with PR4 primers (M and UM) from three samples of each responsive (C4-HI, 59-2-HI), three constitutive (C4-2-HI, 59-HI) and three acquired resistant (C4-HIR, 59-2-HIR) tumors were cloned in competent *E. coli* TOP-10 and five colonies from each sample were sequenced by Macrogen Inc. (Korea). A diagram illustrating the CpG sites that were methylated in one representative sample is shown

was heterogeneous and punctate. In 5azadC-treated 59-HI tumors, only cytoplasmic and perinuclear PRA staining was observed. It may be possible that in the absence of ligand, PRA accumulates in the cytoplasm. Western blots confirmed the increase in PRA expression in both C4-2-HI and 59-HI tumors ($P < 0.01$). The PRB level, on the other hand, was not significantly modified by 5azadC treatment (not shown). Histological evaluation of liver, spleen, and kidney indicated no signs of toxicity due to 5azadC-treatment with the two doses used (0.75 and 1 mg/kg).

Furthermore, in order to test whether 5azadC treatment could modify the antiprogestin response of C4-HIR tumors, in which PRA is silenced by mechanisms other than PRA methylation, these tumors were similarly treated. As expected, no inhibition in tumor growth was observed in these 5azadC- and RU-486-treated mice (Fig. 6).

Constitutive antiprogestin-resistant tumors express high levels of DNA methyltransferases

Dnmt1 and Dnmt3a/b regulate CpG island methylation, and it has been proposed that 5azadC targets Dnmts [35, 36]. Therefore, we were interested in studying Dnmt expression in responsive and resistant antiprogestin tumors.

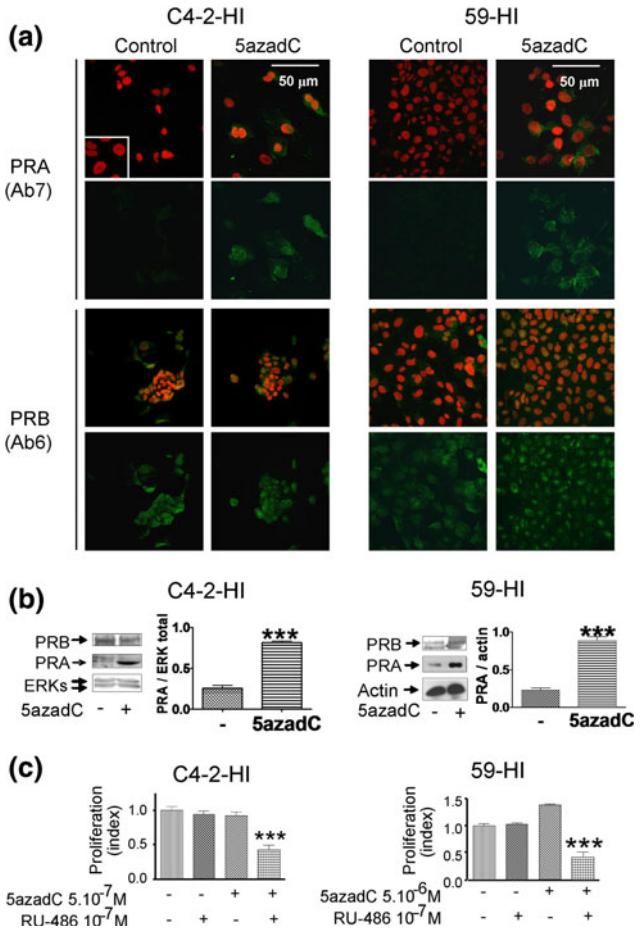

Fig. 4 Treatment of primary cultures of constitutive-resistant tumors with 5azadC and RU-486. **a** PRA (Ab-7) and PRB (Ab-6) expression in control or 5azadC-treated cells. Cells growing in chamber slides in the presence of 10% FCS were treated for 96 h with or without 5azadC (C4-2-HI: 5×10^{-7} M; 59-HI: 6×10^{-6} M) and processed for immunofluorescence. FITC-conjugated secondary anti-mouse antibodies were used. PI was used for nuclear counterstaining. **b** PRA and PRB expression was studied by western blot using Ab-7 and Ab-6 antibodies in whole cell extracts of primary cultures of constitutive-resistant tumors treated with or without 5azadC as described in **a**. PRA expression was quantified in three different blots using different extracts, and an increase in PRA expression was observed in treated cells. *** $P < 0.001$. **c** Effects of 5azadC and RU-486 on (^{3}H)-thymidine uptake. Cells were seeded in 96 microplates and were treated with vehicle, 5azadC, RU-486, or both in the presence of 10% FCS for 48 h. Only 5azadC plus RU-486 inhibited cell proliferation. *** $P < 0.001$ experimental versus control. Proliferation index was calculated as experimental cpm/control cpm (mean ± SEM), and a representative experiment from three using octuplicates in each experiment, is shown

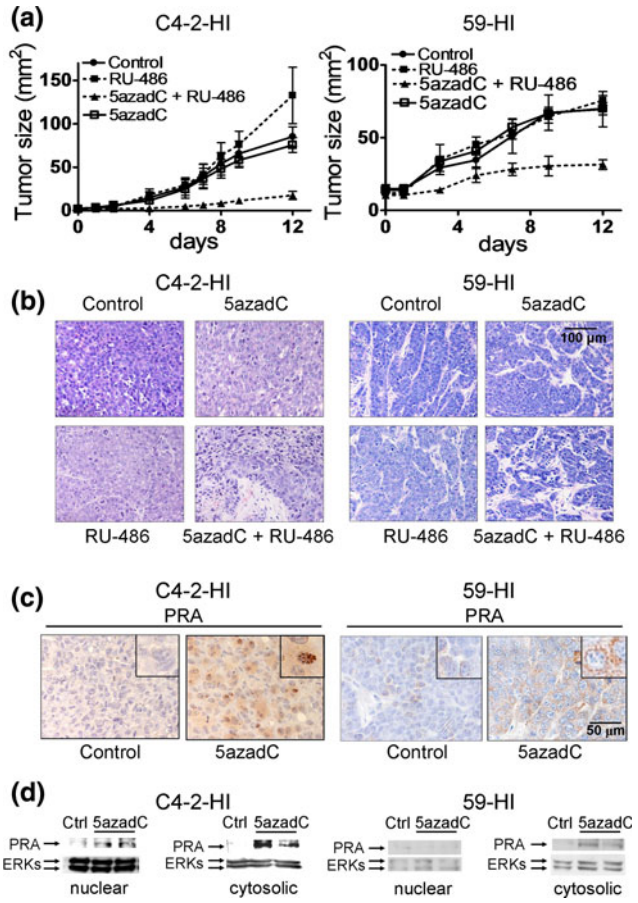

Fig. 5 Treatment of constitutive-resistant tumors in vivo with 5azadC and RU-486. **a** Growth curves. C4-2-HI and 59-HI tumors were transplanted sc into BALB/c female mice. When tumors were palpable, animals (five per group) were treated with vehicle, RU-486 (12 mg/kg/day, s.c.) and/or 5azadC (0.75 mg/kg every other day for C4-2-HI tumors and 1 mg/kg every other day for 59-HI; i.p.). Tumor size was measured every other day (length and width) with a Vernier Caliper, and the mean ± SEM of a representative experiment of the other two is plotted. Inhibition of tumor growth was observed only with combined treatments. **b** Morphological studies. Representative images of H&E-stained slides showing an increase in stromal tissue intermingled with the epithelial nests were observed only in RU-486- and 5azadC-treated tumors. **c** Immunohistochemistry for PRA. Formalin-fixed tumors were processed for immunohistochemistry as described in "Materials and methods" section. The polyclonal rabbit C-19 antibody was used. 5azadC-treated tumors re-expressed PRA. Nuclear and cytosolic staining was observed for C4-2-HI tumors while cytosolic and perinuclear staining was observed for 59-HI tumors (*insets*). **d** Western blots for PRA. Treated and untreated tumors were processed for western blotting as described in "Materials and methods" section. A representative blot of the other three is shown. ERK was used as loading control. An increase in PRA expression was observed in both cytosolic ($P < 0.01$) and nuclear ($P < 0.005$) extracts of 5azadC-treated C4-2-HI tumors, and only in the cytosolic fraction of 5azadC-treated 59-HI tumors ($P < 0.01$)

Immunofluorescence experiments demonstrated that C4-2-HI cells cultured on plastic expressed a higher level of the three Dnmts than the C4-HI cells (Dnmt3a/b, $P < 0.05$; Dnmt1: $P < 0.001$), and that the 59-HI cells showed a higher level of Dnmt3b than the 59-2-HI cells ($P < 0.001$; Fig. 7a). When tumor sections were used, similar results were obtained, although nuclear expression of Dnmt1 and 3a was higher than in cells cultured in plastic, suggesting that tumor microenvironment may be regulating Dnmt activation (Fig. 7a). These studies were corroborated using western blots (Fig. 7b). These data suggest that increased

Table 2 Apoptotic and mitotic indices observed in tumors treated with the combination of RU-486 and 5azadC

	59-HI mean ± SEM		C4-2-HI mean ± SEM	
	Control	5azadC + RU-486	Control	5azadC + RU-486
Apoptotic index	1 ± 0.19	3. 67 ± 0.09***	1 ± 0.22	3. 31 ± 0.02***
Mitotic index	1 ± 0. 069	0.49 ± 0.21*	1 ± 0.08	0.38 ± 0.1*

* $P < 0.05$ and *** $P < 0.001$ treated versus control

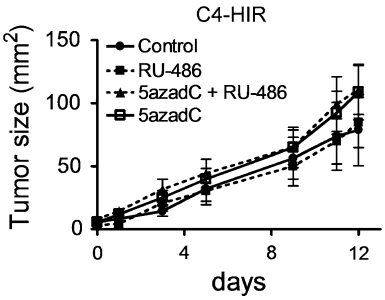

Fig. 6 Acquired resistant tumors do not reverse the resistant phenotype when treated with a demethylating agent. C4-HIR tumors were transplanted s.c. into BALB/c female mice. When tumors were palpable, animals (five per group) were treated with vehicle, RU-486 (12 mg/kg/day, s.c.) and/or 5azadC (0.75 mg/kg every other day; i.p.). Tumor size was measured every other day (length and width) with a Vernier Caliper, and the mean ± SEM of a representative experiment of the other two is plotted. No inhibition in tumor growth was observed in 5azadC- and RU-486-treated mice

levels of Dnmt1 and 3b are related to the increased *PRA* methylation in constitutive-resistant tumors. The greater 5azadC responsiveness of C4-2-HI compared with 59-HI (Figs. 4c, 5a) is consistent with the higher levels of the three Dnmts.

E-Cadherin, p16, PTEN, Rb, and RARβ are not silenced in constitutive antiprogestin-resistant tumors

We were interested in investigating whether the increased Dnmts levels in constitutive-resistant tumors silences other genes that are usually regulated by methylation. Thus, we compared the expression of E-cadherin, p16, Rb, PTEN and RARβ. As observed in Fig. 7c, the expression of these proteins did not follow the same regulation pattern as PRA, demonstrating tumor-specific differences and indicating that silencing of PRA in constitutive-resistant tumors is a specific phenomenon.

Glucocorticoid receptor expression decreases in constitutive antiprogestin-resistant tumors treated with 5azadC

In order to investigate whether 5azadC treatment could be inducing GR expression, and to discard a possible effect of RU-486 mediated by GR, we studied GR expression in C4-2-HI tumors treated with or without 5azadC in vitro and in vivo. The expression of GR decreased in C4-2-HI cells treated with 5azadC (immunofluorescence; Fig. 8a) and in tumors from 5azadC-treated mice (western blots; Fig. 8b), ruling out a possible GR-mediated effect.

Discussion

In previous articles, we have observed a correlation between PRA expression and antiprogestin responsiveness [12]: Antiprogestin-resistant mammary tumors show a lower expression level of PRA than do responsive tumors. Moreover, we have shown that the reacquisition of hormone sensitivity in tumors with acquired resistance was accompanied by the restoration of PRA expression [13]. In this study, we clearly demonstrate that PRA expression is silenced in constitutive antiprogestin-resistant tumors by methylation of the *PRA* promoter. Treatment with a demethylating agent restores PRA expression and antiprogestin responsiveness only in the constitutive-resistant tumors. These tumors showed a high expression levels of Dnmt1 and 3a/b, which may be responsible for the increased *PRA* methylation. The mechanism by which PRA is silenced in tumors with acquired antiprogestin resistance remains to be elucidated.

There exist very few studies regarding PR silencing in breast cancer. In ER- and PR-negative breast cancers, it has been shown that the *PR* promoter is methylated in 39–46% of tumors [21]. Similar findings have been reported by others [34, 35]. More recently, Vasilatos et al. [36] studied a series of genes, among them *PRB* and *PRA*, and have reported that CpG island methylation of *PRA* together with *RARβ* (M4), *INK4α/ARF*, and *HIN-1* may predict non-*BRCA1/2*-associated mammary carcinogenesis and tumor progression. There are, however, no clinical or experimental studies suggesting that *PR* or *ERα* methylation is the possible epigenetic mechanism related to acquired hormone resistance. Our studies clearly demonstrate that different epigenetic mechanisms regulate constitutive and acquired resistance, with CpG methylation of hormone receptor genes being involved in the former.

Most of the studies in experimental models have focused on *ERα* methylation in MDA-MB-231 cells, and PR, most

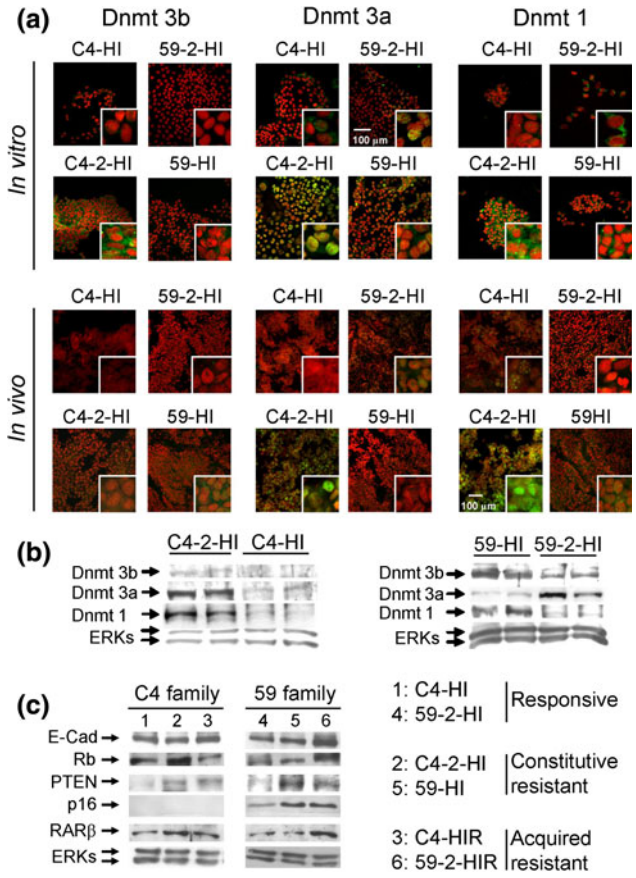

Fig. 7 Expression of Dnmts in constitutive-resistant tumors compared with the responsive tumors and expression of proteins regulated by methylation. **a** *Top* Immunofluorescence in primary cultures. Cells growing in chamber slides with 10% FCS were fixed and immunostained with Dnmt3a-, Dnmt3b-, and Dnmt1-specific antibodies and FITC-labeled secondary antibodies. PI was used for nuclear staining. Staining was quantified as explained in "Materials and methods" section. C4-2-HI cells express a higher level of Dnmt3a and 3b ($P < 0.05$) and Dnmt1 ($P < 0.001$) than C4-HI, and only a higher level of Dnmt3b ($P < 0.001$) was observed in 59-HI compared with 59-2-HI. *Bottom* Immunofluorescence in frozen tumor sections. Frozen sections from the same tumors samples which were analyzed by MSP were immunostained for Dnmt3a, Dnmt3b, and Dnmt1 using the same antibodies described above. C4-2-HI expressed higher levels of Dnmt3a, Dnmt3b, and Dnmt1 ($P < 0.001$) than C4-HI, and 59-HI expressed higher levels of Dnmt3b and Dnmt1 ($P < 0.001$) than 59-2-HI. **b** Representative western blots for Dnmts using nuclear protein extracts. Samples processed for western blots from the same tumors that were used to show PR isoform ratio in Fig. 2 were used to evaluate Dnmts' expression. ERKs were used as loading controls. The band intensities of two different western blots of the different Dnmts were quantified in relation to the loading control. C4-2-HI expressed higher levels of Dnmt3a ($P < 0.001$), Dnmt3b, and Dnmt1 (both $P < 0.01$) than C4-HI. 59-HI expressed higher levels of Dnmt1 ($P < 0.01$) and Dnmt3b ($P < 0.001$) than 59-2-HI. c Expression of other methylation-regulated proteins. The same tumor extracts described above were also probed with antibodies to E-cadherin, Rb, PTEN, p16, and RARβ. The expression pattern of these proteins was different from the PRA expression pattern shown in Fig. 2

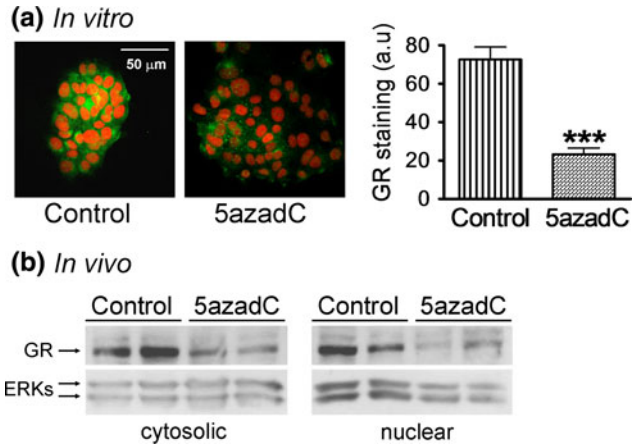

Fig. 8 Gucocorticoid receptor (GR) expression in 5azadC-treated and -untreated constitutive-resistant C4-2-HI tumors. **a** *Left* Cells growing with 10% FCS were treated for 96 h with or without 5azadC (5×10^{-7} M) and processed for immunofluorescence using the GR antibody as described in "Materials and methods" section. FITC-conjugated secondary antibodies were used. PI was used for nuclear counterstaining. *Right* GR staining was quantified using the Image Quant software as described in "Materials and methods" section. 5azadC induced a down regulation in GR expression in vitro; *** $P < 0{,}001$. **b** GR expression was analyzed in the nuclear and the cytosolic extracts from control or 5azadC-treated C4-2-HI samples by western blot. ERKs were used as a loading control

specifically PRB, has been evaluated only as an ERα-regulated gene. In these cells, it has been demonstrated that both ER and PR are silenced by promoter methylation. Treatment with demethylating agents restored ERα and PRB expression, but not PRA expression [19]. The same group demonstrated that ERα was able to restore PR expression even if *PR* was still methylated, since the exogenous transfection of ERα was able to restore PRB expression [37]. However, a different picture has been reported in MCF-7-derived clones. In this system, it has been shown that disruption of ERα signaling alone induces *PR* methylation and that both re-expression of ERα and *PR* demethylation are necessary for PR re-expression [35]. Interestingly, MDA-MB-231 cells treated with demethylating agents in vitro acquired tamoxifen responsiveness [38]. In addition, MDA-MB-435 cells treated in vivo with a combination of Dnmt and HDAC (histone deacetylase) inhibitors showed an inhibition of tumor growth that was more evident in ovariectomized animals [39]. However, to our knowledge, there are no reports examining the in vivo effect of antiestrogens or tamoxifen in combination with demethylating agents.

The strength of our study is that we were able to demonstrate *PRA* methylation for the first time in a mouse model using two different spontaneous constitutive-resistant tumors, and that in both cases antiprogestin responsiveness in vivo could be restored after PRA re-expression. This is important not only in the context of the experiments described herein, but, in addition, they support our hypothesis that high levels of PRA are predictive of

antiprogestin responsiveness. Interestingly, although we have observed an increase in PRA expression in 5azadC-treated tumors, in most cases, the expression level of PRB was still higher than the expression level of PRA. This might be because only some cells re-expressed PRA following treatment with 5azadC, as observed by immunohistochemistry. This heterogeneity may explain why the tumors only showed decreased growth and did not regress completely.

It has been proposed that impaired ER signaling may be enough to induce methylation of ER target genes, among them PR [35]. Since all of these tumors come from hormone-dependent tumors that express a high level of ERα, PRA, and PRB, it is possible that in the hormone-independent switch, these tumors suffered a disruption in ER signaling that, in turn, induced *PRA* methylation. This kind of mechanism has been recently shown in MCF-7 cells cultured in the presence of an ERα-specific siRNA [35]. However, we could not find a pattern demonstrating that constitutive antiprogestin-resistant tumors had lower ERα levels than the responsive tumors. We used four different primer pairs to examine *ERα* promoter methylation without finding any clear differences between the methylation pattern of resistant and responsive tumors (unpublished data). Moreover, we have evidence that PRB is still important for the growth of these tumors, as antisense oligonucleotides targeting PR inhibit cell proliferation (unpublished data). Another possibility is that the increase in the expression of the Dnmts observed in the constitutive-unresponsive tumors may be responsible for methylating several genes that favor MPA-independent tumor growth: among them *PRA*. In support of this hypothesis, all the constitutive-resistant tumors of our model grew faster in vivo and in vitro, showing a stromal-independent pattern of growth. However, when we used 5azadC alone, no significant inhibition of tumor growth was observed. The restitution of suppressor functions by 5azadC treatment should have induced an inhibitory effect per se. Instead, we only observed the inhibitory effect in the presence of RU-486. In contrast, the *E-cadherin*, *p16*, *PTEN*, *Rb*, and *RARβ* genes, all known to be regulated by DNA methylation, did not follow the same pattern of expression as PRA. Taken together, this suggests that these tumors exhibit specific *PRA* methylation.

There is a compelling experimental and clinical evidence indicating that progestins play an important role in the induction and maintenance of the neoplastic phenotype in the mammary gland [40–48]; and thus, the PR may be a valid therapeutic target. In this regard, several studies have demonstrated therapeutic effects of antiprogestins either alone or together with antiestrogens in different experimental models [49–51].

RU-486 is a potent antiprogestin and an antiglucocorticoid [52]. The key role of PR in our experimental model has already been assessed since two antiprogestins with less antiglucocorticoid effects such as onapristone (ZK 98299) and ZK 230211 also induced tumor regression [13, 53]. Moreover, PR antisense oligonucleotides inhibited tumor growth both in vivo and in vitro [54]. However, the possibility that 5azadC treatment would be increasing GR receptors had to be discarded. Interestingly, a decrease in GR expression was observed in 5azadC-treated tumors both in vivo and in vitro, suggesting that the involvement of GR in RU-486-induced inhibitory effect is unlikely.

Demethylating agents have been approved for use in hematologic malignancies, and they are used as differentiating agents [55]. Moreover, it has been proposed that they may decrease the "stemness" of the tumors and increase their differentiation [56]. Our data, together with those of others, suggest that the Dnmt inhibitors may be used temporarily to restore the expression of therapeutic targets [16, 38, 56], in our case PRA.

Carcinoma-associated fibroblasts are key players regulating HI tumor growth in our model [32]. Recently, it has also been shown that these cells are capable of regulating gene silencing in epithelial tumor cells [57]. We were interested in investigating whether carcinoma-associated fibroblasts from tumors with acquired resistance could change the hormone responsiveness of the epithelial cells. Surprisingly, although we did not find any differences between both types of fibroblasts, we observed that epithelial cells with acquired resistance growing on plastic reacquired their hormone responsiveness and PRA expression. This highlights the reversibility of PRA expression, which can be induced by growth on plastic, estrogen treatment [13], and the duration of the absence of the hormone [13], but not by 5azadC treatment in tumors with acquired resistance. This suggests the involvement of different epigenetic mechanisms in the regulation of PRA silencing in acquired antiprogestin resistance.

In summary, we have demonstrated PRA silencing by promoter methylation in constitutive antiprogestin-resistant tumors, and that this increased methylation could be correlated with a high expression level of Dnmts1 and 3b. In vitro and in vivo treatment with a demethylating agent, which was unable to decrease tumor growth, was able to restore PRA expression and antiprogestin sensitivity. These results support a therapeutic role for Dnmt inhibitors in combination with endocrine therapy for those tumors with a high expression level of Dnmts. The correlation between PRA expression and antiprogestin responsiveness supports the use of antiprogestins in breast cancer which should be therapeutically exploited.

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Conflicts of interest statement The authors declare that they have no competing interests.

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