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PRECLINICAL STUDY

Reversal of antiprogestin resistance and progesterone receptor isoform ratio in acquired resistant mammary carcinomas

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Abstract To explore mechanisms related to hormone resistance, three resistant variants of the MPA mouse breast cancer tumor model with low levels of progesterone receptor (PR) isoform A (PR-A)/high PR-B expression were developed by prolonged selective pressure with antiprogestins. The resistant phenotype of one tumor line was reversed spontaneously after several consecutive passages in syngeneic BALB/c mice or by 17-β-estradiol or tamoxifen treatment, and this reversion was significantly associated with an increase in PR-A expression. The responsive parental tumors disclosed low activation of ERK and high activation of AKT; resistant tumors on the other hand, showed the opposite, and this was associated with a higher metastatic potential, that did not revert. This study shows for the first time in vivo a relationship between PR isoform expression and antiprogestin responsiveness, demonstrating that, whereas acquired resistance may be reversed, changes in kinase activation and metastatic potential are unidirectional associated with tumor progression.

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Introduction

At the time of original diagnosis two-thirds of breast cancers express the estrogen receptor (ER), progesterone receptor (PR) or both. This knowledge has led to the development of endocrine therapies aimed at reducing 17-β-estradiol (E_2) activity either by blocking its biosynthesis using aromatase inhibitors (AI) or by creating competition for its binding to the ER using the selective ER modulator, tamoxifen (TAM), or the selective ER disrupter fulvestrant. Despite these therapeutic developments, resistance to all forms of endocrine therapy remains a limiting factor. Comprehensive studies in breast cancer models have led to the concept that cancer tissues dynamically adapt in response to various antihormonal therapies and up-regulate growth factor pathways as a mechanism for resistance [1–3].

Progesterone has been shown to be required for the proliferation of mammary glands [4–6] and mammary carcinomas [7, 8]. The synthetic progesterone, medroxyprogesterone acetate (MPA) behaves experimentally as a mammary-specific carcinogen [9–11], and most importantly, in combination with E_2, it is associated with an increased risk of breast cancer development [12, 13]. PRs exist as two isoforms, known as PR-A and PR-B, which are transcribed from a single gene under the control of distinct promoters [14]. There is increasing evidence that they have different functions in vitro [15] and in vivo [16] and, it has been speculated that differential expression of PR-A and

PR-B is critical for an appropriate mammary gland response to progesterone. Indeed, in transgenic mice carrying an excess of PR-A, mammary gland development is characterized by disproportionate lateral ductal branching, whereas transgenic mice overexpressing PR-B show alterations in lobulo-alveolar growth [17, 18]. Interestingly, PR-A null mice, which only express PR-B, exhibit normal mammary gland development, although they show severe reproductive defects, while PR-B null mice show impaired branching morphogenesis [19]. Taken together, these data suggest that PR-A and PR-B have different functions in different tissues and that the described alterations are related to their relative expression ratios.

Several clinical studies have established that in breast cancer, high total PR levels correlate with an increased probability of response to TAM and longer overall survival. However, tumors with higher PR-A/PR-B ratios were those that relapsed earlier after TAM treatment [20]. Since progestins have been shown to be involved in breast cancer progression, PR antagonists may be effective in blocking the growth of mammary carcinomas expressing a functional PR.

Most studies on the acquisition of hormone resistance in breast cancer have been carried out using experimentally manipulated sub-populations of the human breast cancer cell line MCF-7 [21–23]. However, there are also TAM-resistant cells derived from ZR-75 [24] and T47D [25] and a TAM-resistant model derived from the human xenograft MaCa 3366 [26]. In most of these models, acquired resistance correlated with increased MAPK or AKT activation, suggesting that increased growth factor signaling may bypass steroid hormone receptor function. In addition, these kinases have been shown to activate steroid receptors by ligand-independent mechanisms. There are, however, no available models in which these hypotheses may be directly evaluated in an in vivo syngeneic scenario.

We have developed an experimental model in BALB/c mice in which mammary carcinomas express high levels of the ER and PR and transit through different stages of hormone dependency [9, 10, 27]. This model has proven to be suitable for studying mechanisms related to the acquisition of hormone independence, hormone resistance and tumor regression [28, 29]. MPA-induced mammary tumors are hormone-dependent but several independent variants have been developed [30]. Most of these tumors regress with antiprogestin treatment, although some de novo resistant variants are available. Here we report for the first time the development of three acquired antiprogestin-resistant variants by selective pressure with RU-486. Both de novo and acquired resistant tumors showed an inverted PR isoform ratio as compared with responsive tumors, suggesting that tumors with higher levels of PR-A may constitute a subgroup responsive to antiprogestins. In addition, we demonstrated that only acquired resistance could be reverted, either by successive transplantations in untreated animals or by treatment with E_2 or TAM. Acquired hormone resistance was accompanied by an increase in metastatic potential, as well as increased activation of ERK1/2, and decreased activation of AKT. However, none of these features reverted, and therefore none are associated with hormone responsiveness.

Materials and methods

Animals

Two-month-old virgin female BALB/c mice (IBYME Animal Facility) were used. Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [31].

Tumors

C4-HD is a mammary tumor induced by medroxyprogesterone acetate (MPA) in a BALB/c female mouse [30] that is maintained by serial subcutaneous (sc) transplantations into MPA-treated female mice. Two hormone-independent (HI) variants were generated: C4-HI and C4-2-HI. C4-HI grew in a mouse that was not treated with MPA; it expresses ER and PR and regresses with E_2 or antiprogestin treatment [30]. C4-2-HI, the second variant to arise, showed a de novo estrogen and antiprogestin resistance. 59-HD is also an MPA-induced mammary carcinoma [32], which gave rise to two HI variants: 59-HI and 59-2-HI. 59-HI, proved to be a de novo resistant tumor, 59-2-HI regressed completely with E_2 or antiprogestin treatment [33, 34].

Tumors smaller than 100 mm^2 growing in untreated female mice were excised and immediately frozen at −80°C for western blots or immunofluorescence studies. To compare the metastatic potential of each tumor line, animals were euthanized 55 days after being inoculated. The presence of metastases was histologically confirmed.

Generation of tumors with acquired resistance to antiprogestin treatment

Tumors were transplanted as described above. When the tumors reached a size of 50 mm^2, RU-486 (Sigma Co., St Louis, MI) silastic pellets (6 mg) were implanted sc at the back of the animals. C4-HI and 59-2-HI regressed almost completely, but occasionally one tumor started to grow slowly in RU-486 treated mice after one month of treatment. To further select the resistant phenotype, these tumors were transplanted again into RU-486 treated mice

until a similar growth rate was obtained between treated and untreated animals. These new tumor variants, resistant to the antiprogestins RU-486, were named C4-HIR and 59-2-HIR, respectively, and maintained by syngeneic transplantation into two RU-486-treated and two untreated mice. Tumors growing in RU-486 treated mice were used for further syngeneic transplantations.

Reversal of the antiprogestin resistant phenotype

To investigate whether acquired resistance was a reversible phenomenon, C4-HIR tumors growing in untreated mice were chosen for the next passages. These tumors were implanted in two RU-486-treated and two untreated mice and tumors growing in untreated mice were chosen for the next passages. When the tumors arrested their growth after RU-486 administration it was consented that this variant had reverted its resistant phenotype and become antiprogestin sensitive, this variant was named C4-HIRev. Tumors growing in RU-486 untreated mice were used for further syngeneic transplantations.

Effect of RU-486, E_2, ZK 230211 (ZK) and TAM on tumor growth

C4-2-HI, C4-HI and C4-HIR tumors were transplanted in syngeneic mice as explained above and measured every 2 days with a Vernier caliper (length and width). Treatments were initiated when tumors reached a size of 50 mm^2. ZK 230211(Bayer Schering Pharma AG, Berlin) and RU-486 were inoculated sc in doses of 12 mg/kg/day, and tamoxifen citrate (Laboratorios Gador, Buenos Aires) in doses of 5 mg/kg/day. E_2 (Sigma Co) silastic pellets (5 mg) were implanted sc in the back of the animals. All experiments were repeated twice using at least, four mice per group. Tumors were processed for histologic evaluation. Paraffin sections were stained with Hematoxilin-eosin. Sections were analyzed using a Nikon Eclipse E800 Microscope with ACT-2U (for Nikon) software.

Effect of RU-486 and E_2 on ERα and PR expression

C4-HI and C4-HIR tumors were treated as described above. After 2 days animals were euthanized, tumor samples taken and either processed for histological evaluation, or frozen at −80°C for western blots or immunofluorescence studies.

Western blots

Total or nuclear extracts were processed for western blotting as described previously [33]. The membranes were then incubated with PR (C-19, sc 538 Santa Cruz Biotech. Inc, Santa Cruz, CA), PR-B (Ab 6, Neomarkers, Lab Vision Corp, Fremont, CA), ERα (MC-20, Santa Cruz), ERK (sc-94 Santa Cruz), p-ERK (sc-7383 Santa Cruz), AKT (Cat. 610837, BD Transduction Laboratories), p-AKT (Ser 473 9271, Thr 308 4056; Cell Signaling Tech, Danvers MA), over night (ON) at 4°C, at 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).

Immunofluorescence

Frozen sections were incubated with anti PR-A Ab (C-19), which by this technique only detects PR-A, dissolved in blocking buffer at a 1/100 dilution ON at 4°C, and then incubated with anti-rabbit FITC (FI-1000, Vector Laboratories Burlingame, CA. 1/100 dilution) secondary antibodies for 1 h at room temperature. The nuclei were stained with either propidium iodide (PI, Sigma) or 4′,6-diamino-2-phenylindole (DAPI, Sigma) and mounted with Vectashield (Vector Laboratories). Sections were analyzed under a Nikon Eclipse E800 Confocal Microscope using EZ-C1 2.20 software. DAPI was visualized using the fluorescence lamp.

Effects on metastasis

To compare the metastatic potential, animals were euthanized 55 days after inoculation. Axillary lymph nodes and lungs were fixed and the presence of metastases confirmed by histological examination.

Statistical analysis

Western blot band intensity or cell staining were quantified using the Image Quant® software. ANOVA and the Tukey multiple post *t* test were used to compare means of multiple samples; the Student's *t* test was used to compare means of two different groups. Tumor growth curves were studied using regression analysis and slopes compared using ANOVA followed by parallelism analysis. Data analysis was made using Graph Prism 4.0 software.

Results

PR isoform expression in antiprogestin responsive (C4-HI) versus de novo-resistant carcinomas (C4-2-HI)

A typical growth curve for C4-HI tumors is shown in Fig. 1a, left. RU-486, E_2 or RU-486 + E_2 treatment was initiated when the tumors reached 50 mm^2 and these treatments induced significant regression after approximately 10 days ($P < 0.001$). In contrast, C4-2-HI tumors continued growing after treatment (de novo-resistant

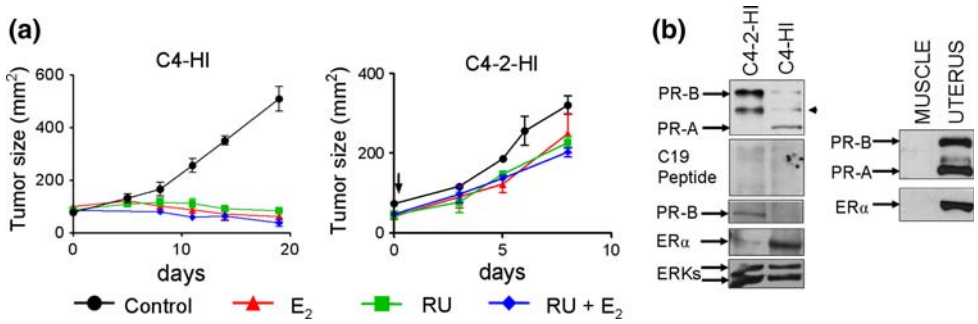


Fig. 1 C4-HI (sensitive) and C4-2-HI (de novo-resistant) tumors have different responses to RU-486 and E_2 and differ in their PR isoform expression. (**a**) Tumor growth curves for C4-HI and C4-2-HI. Tumors were transplanted sc into BALB/c mice and hormone treatments were started when tumors reached a size of approximately 50 mm^2. E_2 was administered as 5 mg silastic pellets implanted sc; RU-486 (RU) was administered as sc injections at doses of 12 mg/day/kg body weight. Only C4-HI regressed when treated with RU or E_2 or a combination of both. Width and length of the tumors were measured, and the tumor area was plotted ($x \pm$ SEM), considering as day 0 the day on which treatments were initiated, (**b**) ERα and PR expression. Representative western blot of the PR using nuclear protein extracts from C4-2-HI and C4-HI tumors. The responsive tumor showed higher levels of PR-A (83 kDa) than PR-B (115 kDa) and the opposite was observed with the unresponsive tumor ($P < 0.001$). Tumors also showed an intermediate band of 105 kDa (arrowhead) when probed with the C-19 Ab. Pre incubation with the blocking peptide abolished all bands. Ab 6, which only recognizes PR-B, also showed higher levels of PR-B in the unresponsive tumor ($P < 0.05$). ERα expression (MC-20 Ab) paralleled PR-A expression ($P < 0.001$; $n = 3$)

pattern; Fig. 1a, right), as untreated tumors did. When immunoblotted, both responsive and resistant tumors showed three bands with the C-19 PR Ab: PR-B (115 kDa), PR-A (83 kDa) and an additional band at 105 kDa (arrowhead). In the responsive tumor C4-HI, the PR-A level was higher than the PR-B level; whereas the opposite was observed in C4-2-HI ($P < 0.001$). All bands disappeared when the antibody was pre-incubated with saturating concentrations of the neutralizing peptide (Fig. 1b, left). PR-B expression detected with the specific PR-B antibody (Ab 6) showed higher ($P < 0.05$) levels of PR-B in unresponsive tumors. In addition, responsive tumors had higher levels of ERα ($P < 0.001$; Fig. 1b, left), with total ERK shown as a loading control. Uterus and muscle were used as positive and negative controls, respectively. As shown in Fig. 1b, right, the intermediate band of 105 kDa was not observed in these tissues. These results suggest that the ratio of PR-A/PR-B can be associated with changes in hormone responsiveness and that high PR-A levels correlate with higher ERα levels.

Development of a tumor variant with acquired antiprogestin resistance

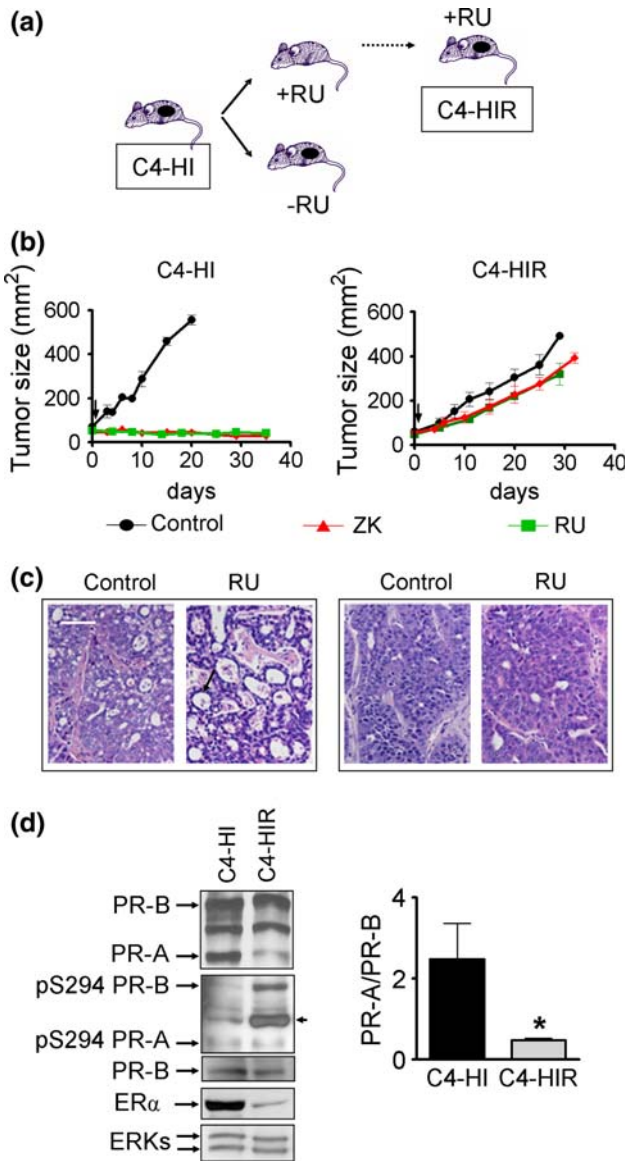
To further investigate whether the ratio of PR-A/PR-B correlated with antiprogestin responsiveness, we developed a resistant tumor by selective pressure under RU-486 treatment. After 20 days, one of the C4-HI isografts started to grow and was named C4-HIR (Fig. 2a). ZK was used to investigate whether the tumor was also resistant to other antiprogestins. As such, C4-HI was sensitive to and C4-HIR was resistant to both antiprogestins (Fig. 2b).

C4-HI tumors are ductal carcinomas with different degrees of differentiation. After 48 h of antiprogestin treatment we observed increased glandular differentiation (Fig. 2c, left). This was associated with a significant reduction in the mitotic index (98%) while the apoptotic index was similar to that of the untreated C4-HI group. C4-HIR tumors were less differentiated than C4-HI, but a slight increase in differentiation was observed after 48 h of RU-486 treatment, which was associated with a 66% of inhibition of mitotic activity (Fig. 2c, right). As before, no changes in the apoptotic indices were observed.

As expected, the PR-A/PR-B ratio was lower in the acquired resistant C4-HIR tumor than in the sensitive C4-HI tumor indicating that we were able to experimentally reproduce the hormone-resistant PR pattern of C4-2-HI tumors (Fig. 1b). PR-B expression, as evaluated by the Ab 6 antibody, was similar or even higher in resistant tumors (Fig. 2d). Interestingly, the expression of ERα paralleled that of PR-A, as observed previously in the C4-2-HI (Fig. 1b). C4-HIR, however, showed increased pSer 294 PR-B and a 94 kDa band (Fig. 2d), suggesting that PR-B is constitutively more activated in resistant tumors. The p94 kDa band follows the same expression pattern as PR-B, suggesting that it may be PR-B-derived. These results show that resistance to antiprogestins correlates with a loss of PR-A, and an increase in PR-B.

Acquired resistance and PR isoform expression is a reversible phenomenon

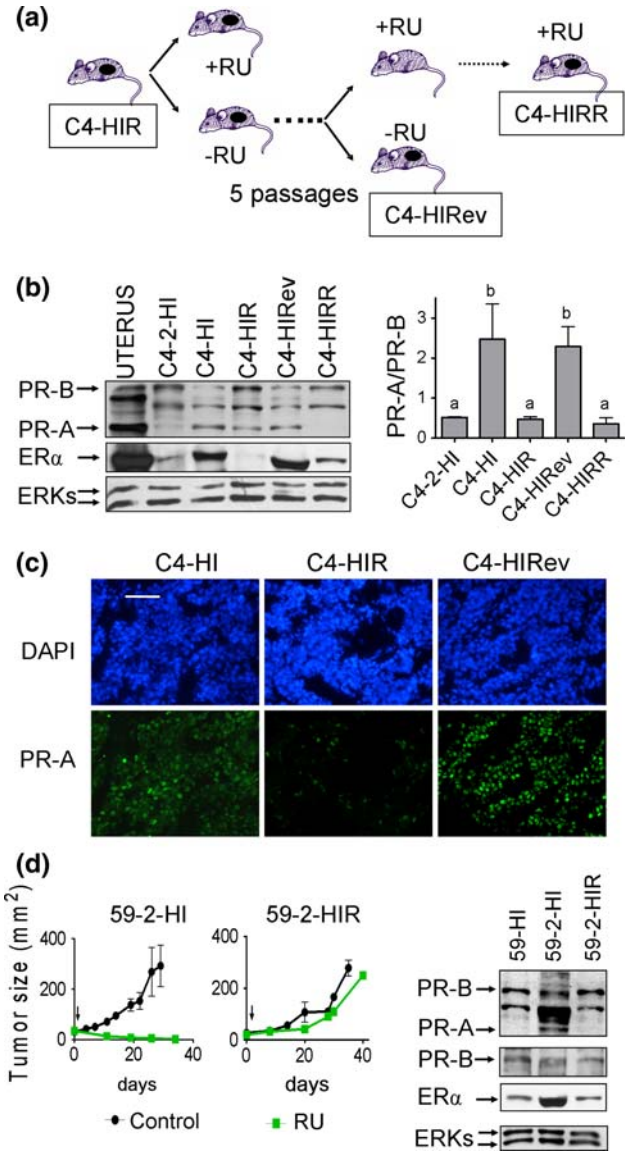
To determine whether antiprogestin resistance could be reversed, C4-HIR tumors at passage eight were transplanted



◀ **Fig. 2** Acquired-resistant tumors show a pattern of PR isoform expression similar to de novo-resistant tumors. (**a**) Generation of an acquired antiprogestin-resistant tumor variant, C4-HIR. C4-HI tumors regress when treated with RU-486 (RU). After 20 days of treatment, one tumor started to grow in the presence of RU and was named C4-HIR. Its hormone resistance was assessed in further transplants in syngeneic mice. (**b**) RU or ZK 230211 (ZK) exert similar effects. C4-HI and C4-HIR showed similar tumor growth responsiveness to RU and to ZK. Tumors were transplanted sc, and treatments with RU, ZK or vehicle were started when tumors reached a size of about 50 mm^2. Both antiprogestins were administered daily as sc injections of 12 mg/kg body weight. Width and length were measured with a caliper and tumor area was plotted ($x \pm$ SEM), considering as day 0 the day in which treatments were initiated. (**c**) Histological features of C4-HI and C4-HIR tumors. Paraffin-embedded sections of tumors were excised 48 h after RU treatment (H&E). RU-treated C4-HI tumors showed an increased glandular differentiation while C4-HIR tumors experienced virtually no histological changes. The arrow shows one glandular structure; bar: 100 μm. (**d**) PR and ERα expression in C4-HI and C4-HIR tumors: nuclear protein extracts were analyzed by western blots. PR-A and PR-B bands were quantified, and the PR-A/PR-B ratio was calculated. The mean ± SEM of four samples of each tumor type is shown (right). C4-HI showed higher levels of PR-A (83 kDa) than of PR-B (115 kDa), while the opposite was observed in C4-HIR tumors ($P < 0.001$). ERα (66 kDa) expression paralleled PR-A expression patterns while similar PR-B levels were observed in the two tumor variants using the Ab 6 Ab. ERKs were used as a loading control. The pSer 294 Ab (Ab 12) indicated that PR-B is highly phosphorylated in the resistant variant. A 94 kDa band followed the same trend as PR-B (arrowhead)

into four mice, two of which received RU-486 treatment as a control to check antiprogestin resistance. Tumors from untreated mice were selected for successive passages (Fig. 3a). After five passages in untreated mice, these tumors recovered their sensitivity to RU-486 and were named C4-HIRev. To further select a resistant phenotype from this new sensitive variant, we proceeded as is documented in Fig. 2 and obtained, by selective pressure under RU-486 treatment, a new resistant tumor named C4-HIRR.

Higher PR-A than PR-B levels, as in the original C4-HI tumors were observed in the acquired sensitive tumor C4-HIRev (Fig. 3b). On the other hand, the de novo-resistant tumor, C4-2-HI, as well as the two tumors with acquired resistance (C4-HIR and C4-HIRR), had the opposite pattern: higher PR-B than PR-A levels. ERα expression followed the same pattern as PR-A in C4-HI, C4-HIR and C4-HIRev. Similar results were obtained by immunofluorescence using frozen sections. PR-A expression, which is the only isoform detected by the C-19 Ab with this technique, was mostly observed in C4-HI and C4-HIRev tumors (Fig. 3c). Taken together, these results indicate that hormone resistance can be a reversible phenomenon and furthermore, they show that a low PR-A/PR-B ratio is a common characteristic of three tumors that are resistant to antiprogestin treatment (C4-2HI, C4-HIR and C4-HIRR). To extend these results to other series of tumors we used the '59' family of MPA-induced mammary carcinomas: the 59-2-HI tumor, which is sensitive to antiprogestin treatment, and 59-HI, which is antiprogestin-resistant (not shown). We have previously demonstrated that PR expression in the de novo resistant tumor 59-HI was similar to that in the de novo-resistant tumor C4-2-HI [33]. Using the same experimental design mentioned above, we generated an acquired resistant tumor that grows in the presence of antiprogestins (59-2-HIR; Fig. 3d). This resistant variant has a growth rate slower than that of the parental tumor 59-2-HI (6.646 ± 1.176 mm^2/day vs. 9.299 ± 1.138 mm^2/day; $P < 0.05$, respectively). Interestingly, the acquired-resistant tumor (59-2-HIR) had a PR isoform expression pattern similar to that of the de novo-resistant tumor (59-HI). Similar PR-B levels were observed using both the C-19 and the specific PR-B antibody, Ab 6. As previously described, ERα expression was higher in the



◀ **Fig. 3** Hormone resistance and PR-A expression are two reversible phenomena. (**a**) Reverting antiprogestin tumor resistance. C4-HIR was transplanted in each passage into four BALB/c mice, two of which were then treated with RU to test their hormone sensitivity. Tumors growing in untreated mice were selected for the next passages. After five passages, the tumors recovered the sensitive phenotype. This tumor was named C4-HIRev. In passage 18, one tumor started to grow again in the presence of RU and was referred to as C4-HIRR. (**b**) Western blot showing PR expression in all C4-HI variants and in the de novo-resistant tumor C4-2-HI. PR-A (83 kDa) and PR-B (115 kDa) bands were quantified and the PR-A/PR-B ratio was calculated in nuclear extracts from each tumor. The mean ratio ± SEM of four samples for each tumor is shown. All sensitive variants showed a high ratio of PR-A/PR-B while the two acquired-resistant variants and the de novo-resistant tumor showed a low PR-A/PR-B ratio; a vs. b: $P < 0.001$. (**c**) Immunofluorescence showing PR-A expression in the sensitive, resistant and reverted tumors. Cryostat frozen sections from C4-HI, C4-HIR and C4-HIRev tumors were immuno stained using the PR-A antibody (green confocal image). Nuclei were counterstained with DAPI (blue non-confocal image). PR-A was down regulated in the resistant variant ($P < 0.01$) and was re-expressed in the reverting tumor; bar: 100 μm. (**d**) PR-A/PR-B ratio in an acquired resistant tumor of another family of MPA-induced mouse mammary tumors. The 59-2-HI is a sensitive tumor that gave rise to the 59-2-HIR variant by selective pressure, while 59-HI is a de novo-resistant tumor**.** Left: Tumor growth curves of 59-2-HI and 59-2-HIR in the presence of RU-486. Tumors were transplanted sc and hormone treatments were started when tumors reached a size of about 50 mm^2. RU-486 (RU) was given in daily sc injections at doses of 12 mg/kg body weight. Right: PR expression. Representative western blot of PR using nuclear extracts from 59-2-HI, 59-HI and 59-2-HIR. The resistant tumors showed almost no detectable PR-A. Using the Ab 6 Ab which only recognizes PR-B, no differences were observed between the three tumor variants. Staining for ERKs was used as loading control

responsive tumor 59-2-HI. These results corroborate our hypothesis that changes in PR isoform expression patterns may be predictive of hormone responsiveness. The low PR-A/PR-B ratio is now a common characteristic of the five tumors that are resistant to antiprogestin treatment (C4-2HI, C4-HIR, C4-HIRR, 59-HI and 59-2-HIR).

The biological features of resistant tumors are different from the parental tumors and do not revert

To further characterize biological parameters of the different C4 tumor variants, we compared the presence of axillary lymph node metastasis, as well as the growth rate of the sensitive C4-HI, C4-HIRev and resistant C4-HIR tumors. As observed in Fig. 4a, increased axillary lymph node metastases were observed in resistant and reverted tumors ($P < 0.05$).

Surprisingly, these two tumors demonstrated a slower growth rate than C4-HI. A similar phenomenon was observed in the 59 tumor family, in which the resistant variant 59-2-HIR had a slower growth rate than the sensitive tumor (59-2-HI). Thus, aggressiveness in this tumor model, was related to the metastatic potential rather than to the rate of tumor growth. These data therefore suggest that although C4-HIRev regained its sensitivity to antiprogestins and increased PR-A expression, the increased aggressiveness observed in the C4-HIR tumors was not reversible. These data show that progression in this model is not reversible and is independent of hormone responsiveness.

AKT and ERK activation and hormone resistance

The AKT and MAPK pathways have both been related to ligand-independent activation of steroid receptors leading to hormone resistance, and activation of metastatic cascades [35]. Since we have a suitable model for hormone resistant tumors which have acquired simultaneously a more metastatic, hormone-resistant phenotype, whereas reverted tumors only maintain the metastatic phenotype, we decided to evaluate the role of ERKs and AKT activation in these tumors. As observed in Fig. 4b, the parental C4-HI tumor showed low

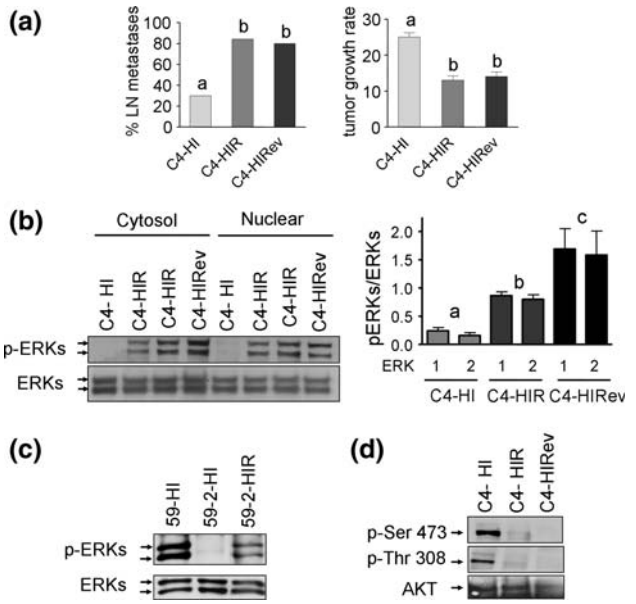


Fig. 4 The metastatic phenotype, growth rate and kinase activation patterns are not reversed. (**a**) Left: The percentage of animals bearing axillary lymph node metastasis (LN metastasis) at autopsy after 55 days of transplantation is shown; a vs. b: $P < 0.01$. Right: Tumor growth rate, expressed as mm^2/day, was higher in C4-HI and lower in C4-HIR and C4-HIRev; a vs. b: $P < 0.001$. (**b**) ERK activation levels in cytosolic and nuclear fractions of the C4 tumor family. All variants, except C4-HI, showed high levels of pERK in western blots a vs b and c: $P < 0.001$. (**c**) ERK activation levels in nuclear fractions of the 59 tumor family. The two resistant tumors showed high pERK levels in western blots; $P < 0.001$. (**d**) AKT activation correlates with tumor growth. Tumor nuclear extracts from the three different C4 variants were analyzed by western blot using p-AKT and total AKT antibodies. High levels of p-Ser 473 and p-Thr 308 AKT were only observed in the parental C4-HI tumor ($P < 0.001$)

levels of pERKs in both cytosoloic and nuclear fractions. All other variants, regardless of their hormone sensitivity, showed high levels of activated ERK 1 and 2. Similarly, the parental 59-2-HI tumor showed low pERKs, while the acquired and the de novo-resistant tumors showed high levels of pERKs (Fig. 4c). Interestingly, the C4-HI tumor, which showed low levels of p-ERKs, was the only one to show high p-AKT levels (Fig. 4d). Thus, in this tumor model, the changes in kinase activation observed during the transition to the antiprogestin-resistant phenotype are not related to the ability of tumors to respond to hormones, but are more likely the consequence of the selection of more aggressive clones.

In addition, since AKT was activated in tumors with a higher growth rate and lower metastatic ability, a correlation between pAKT and growth rate may also be suggested.

PR-A/PR-B ratio can be pharmacologically manipulated

Clinically, the reversion of the resistant phenotype with time is not a choice. However, we reasoned that, if the PR

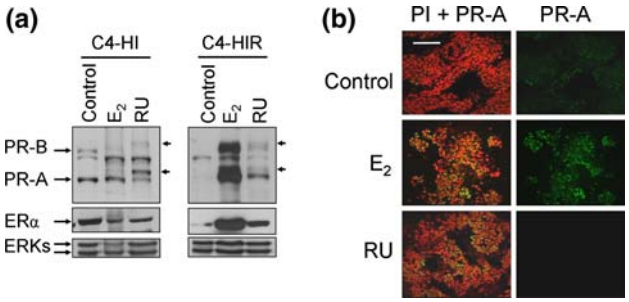


Fig. 5 Re-expression of PR-A after hormonal treatment. (**a**) Western blots. Nuclear protein extracts from C4-HI and C4-HIR tumors treated with E_2 or RU-486 (RU) for 48 h were analyzed by western blots. A significant increase in PR-A and PR-B expression was observed after E_2 treatment in C4-HIR tumors. RU-486 increased PR-A and up-shifted bands are shown with arrow heads. ERα (66 kDa) was downregulated in C4-HI and up regulated in C4-HIR after hormonal treatment. Total ERKs were used as a loading control. Here we show a representative western blot from a total of four using different tumor extracts. (**b**) Immunofluorescence of PR-A. Frozen sections of C4-HIR treated for 48 h with E_2 or RU-486 were immunostained with the polyclonal PR-A Ab (green). Propidium iodide (PI) was used as nuclear counterstaining (red); bar: 120 μm. Images were obtained using a confocal Nikon Microscope

isoform ratio is responsible for the sensitivity to antiprogestins, then re-expression of PR-A would influence such a response. To evaluate whether PR-A expression could be pharmacologically restored to primitive levels, antiprogestin-sensitive (C4-HI) and -resistant (C4-HIR) tumors were treated for 48 h with E_2, which is known to upregulate PR expression, or with RU-486. The expression of PR isoforms was then evaluated by western blot. In C4-HIR, E_2-treatment induced a significant upregulation of both PR isoforms, whereas RU-486 induced a slight increase in PR-A over PR-B (Fig. 5a, left). In C4-HI tumors, on the other hand, RU-486 induced an up-shift in PR-A and in PR-B. These shifted bands after RU-486 treatment have also been observed in other responsive tumors treated with RU-486 [36]. Curiously, E_2 induced a downregulation of ERα in C4-HI and an upregulation in C4-HIR. Western blot data were confirmed by immunofluorecence (Fig. 5b) and by immunohistochemistry (not shown). These results indicate that PR isoform expression can be pharmacologically manipulated to restore the PR-A/PR-B ratio.

Resistant tumors may become sensitive to antiprogestins when treated with E_2 or TAM

To investigate whether resistant tumors could re-acquire RU-486 sensitivity after E_2 or TAM treatment, C4-HI and C4-HIR tumors were transplanted sc in BALB/c mice and treated with RU-486 alone or in combination with E_2 or TAM. Controls received vehicle, E_2 or TAM alone. C4-HI tumors showed a similar inhibitory response with E_2, RU-486 and RU-486 + E_2 during the first 30 days after the

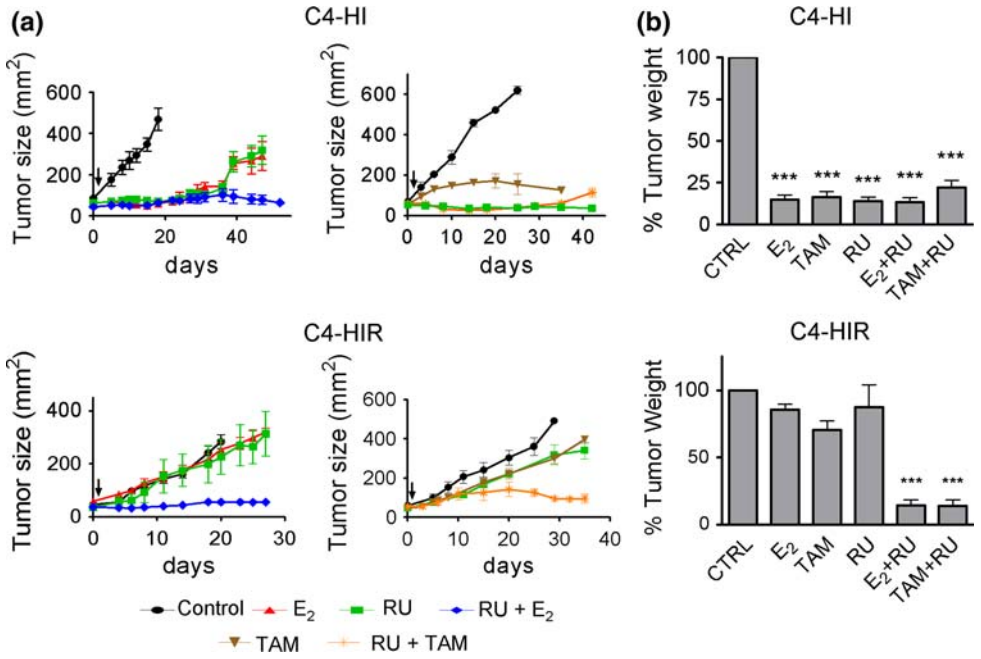


Fig. 6 (**a**) Treatment with E_2 or TAM in vivo restores RU-486 sensitivity: Growth curves. Mice were treated with E_2, RU-486, E_2 + RU-486, TAM or TAM + RU-486. Treatments started when tumors reached a size of approximately 50 mm^2. Controls were treated with vehicle. Width and length were measured with a caliper and tumor area was plotted ($x \pm$ SEM) considering as day 0 the day in which treatments were initiated. (**b**) Tumor weight. In a similar experiment, mice were euthanized 20 days after the treatments were initiated and tumors were weighed. The media of tumor weight in the control group was considered to be 100%. Combined treatments produced a decrease in tumor weight as compared to single treatments only in C4-HIR (*** $P < 0.001$)

initiation of the treatments. After that, tumors from RU-486 or E_2-treated mice started to grow while tumors from E_2 + RU-486-treated mice remained quiescent (Fig. 6a). TAM induced an inhibition of tumor growth ($P < 0.001$) although no benefits were observed in combined treatments as compared with single treatments. On the other hand, although single treatments with RU-486, E_2 or TAM induced slight or no changes in C4-HIR tumor growth, the combination of RU-486 with E_2 or TAM induced an impressive inhibition of tumor growth (Fig. 6a). In other experiments, mice were euthanized 20 days after treatment was initiated. Tumors were weighed and C4-HIR tumor weight was found to be significantly smaller after combined treatments than in controls or single-treated animals (Fig. 6b). When tumors were histologically analyzed, signs of tumor regression were observed (Fig. 7). C4-HI treated with RU-486 + E_2 showed almost complete regression. Thus only a few residual tumor cells surrounded by an abundant extra cellular matrix and fibroblasts were observed. C4-HIR tumors treated with RU-486 + E_2 or TAM showed few tumor nests with glandular differentiation (arrow) surrounded by a conspicuous stroma (dotted arrow; Fig. 7).

These results indicate that hormone-resistant tumors may recover endocrine sensitivity and that even highly metastatic tumors may regress if appropriately treated with a suitable hormone therapy.

Discussion

We have previously reported that sensitive and de novo-resistant mammary carcinomas of our model had similar levels of PR as evaluated by RNAse protection assays and by immunohistochemistry, although they displayed different levels in western blots [33]. We now report that the difference between resistant and responsive tumors resides mostly in the PR isoform ratio. We have also extended these findings to acquired resistant tumors which, interestingly, share the same PR isoform ratio as the de novo-resistant tumors, i.e., lower expression of PR-A than PR-B isoform. In this study we have used C-19 Ab which stains only PR-A in inmmunofluorescence, and both PR isoforms in western blot studies [37], observing a reduction in PR-A expression in the resistant variants. An intermediate band located just under PR-B, was also observed. This band of 94 kDa may be mistaken for PR-B. Only 8% SDS-PAGE gels are able to separate this band from the PR-B of 115 kDa. Samples resolved in 10 or 12% SDS-PAGE gels show that these bands come together, yielding PR-A/PR-B ratios that may distort the ratio calculated using 8% SDS-PAGE. As observed in Fig. 1, all bands disappeared with incubation of excess synthetic peptide. Studies using the Ab 6 antibody, which stains only PR-B, confirmed that PR-B levels were not decreased in resistant tumors.

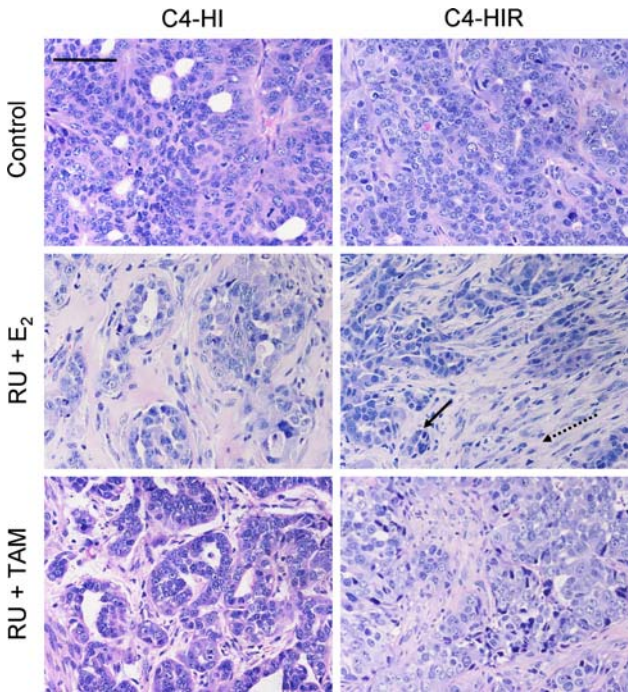


Fig. 7 Treatment with E_2 or TAM in vivo restores RU-486 sensitivity: Histological evaluation of tumors. Tumor sections from control or treated mice at the end of the experiment. C4-HI: control; packed group of neoplastic cells showing glandular differentiation with occasional mitosis. Stroma is scant or absent. (E_2 + RU-486)-treated; the neoplastic proliferation is composed of well-defined glandular structures embedded in a dense collagenous stroma. (TAM + RU-486)-treated; malignant neoplastic proliferation composed of irregular glands, lined by loosely cohesive epithelial cells. The glands are immersed in a sparse stroma. C4-HIR: control; malignant proliferation showing few and irregular glandular structures with abundant atypia and mitoses. The stroma is scant and dense. (E2 + RU-486)-treated; groups or islands of malignant neoplastic cells embedded in a dense collagenous stroma (dotted arrow). In some areas, glandular differentiation is evident (arrow), but most of the proliferation is solid. (TAM + RU-486)-treated; poorly differentiated adenocarcinoma with a few irregular glands composed of a highly heterogeneous cell population. The stroma is dense and relatively abundant (H&E, bar: 120 μm)

Our results are in agreement with the hypothesis that high levels of PR-A expression could be a marker of antiprogestin responsiveness, and this is in accordance with reported data indicating that the antiprogestin RU-486 may display agonistic effects when bound to PR-B, and antagonistic effects when bound to PR-A [38].

It has also been proposed that RU-486 may have inhibitory effects on tumor cell growth by mechanisms other than those mediated by PR. In our tumor model, we have already demonstrated that the antiprogestin onapristone was as effective as RU-486 at inducing regression of 59-2-HI, 32-2-HI and C7-2-HI tumors [36] and, furthermore, that antisense oligonucleotides of PR inhibited 32-2-HI tumor growth [39]. In the present study, we have also shown that the new antiprogestin ZK 230211 is as effective as RU-486 in inducing tumor regression. These data, together with the experimental data demonstrating that the antiprogestins also inhibited cell proliferation in primary cultures [40], indicate a PR's direct mediating effect on tumor growth. In addition, we demonstrated that RU-486 resistant tumors had cross-resistance to ZK suggesting that the presence of the PR-A isoform is mandatory for antiprogestin responsiveness. It is interesting to remark, however, that although tumors with acquired or de novo antiprogestin resistance do not regress with antiprogestin treatment, a slight decrease in tumor growth rate may be observed in some cases. This slight inhibitory effect might be due to systemic effects of antiprogestins such as regulation of NK cells [41], or to systemic mechanisms related with antiglucorticoid properties [38].

Down regulation of PR-A in C4-HIR and in 59-2-HIR resistant tumors was accompanied by down regulation of ERα expression giving rise to the possibility that the decrease in ERα expression could be also responsible for the acquired antiprogestin resistance. However, the fact that the third acquired resistant variant, C4-HIRR, shows low PRA levels without significant changes in ERα levels, highlights the significance of the PR isoform ratio in the onset of the antiprogestin resistant phenotype.

We have previously shown that in our model all de novo antiprogestin-resistant tumors were also resistant to estrogens. Interestingly, before the use of TAM [42] postmenopausal women with ER-positive breast cancer were treated with high doses of estrogens, such as diethylstilbestrol or ethinyl estradiol [43, 44], which induced tumor regression. High doses of diethylstilbestrol are still today routinely recommended to postmenopausal women who have failed multiple endocrine therapies [45].

Different mechanisms have been proposed to explain hormone resistance. As TAM is the most widely used agent in breast cancer therapy, hormone-resistance was for years, almost synonymous with TAM-resistance. With the increasing use of AI in the last few years, studies regarding AI-resistance are now available. However, there is to our knowledge no study regarding antiprogestin-resistance in breast cancer. The main reason is that despite all the increasing evidence relating PR with proliferative signals in the mammary gland [4, 6, 46], there are very few studies focused on unraveling the role of PR in breast cancer.

Clinical tumors which become hormone resistant are also more metastatic, leading to the hypothesis that hormone resistance and metastatic ability are related phenomena during tumor progression. The data reported herein indicate that this is not always necessarily true. Moreover, they address an important issue in hormone resistance: the possibility of reverting the resistant phenotype. Our results suggest that epigenetic mechanisms may be involved in regulating PR-A expression, which may be

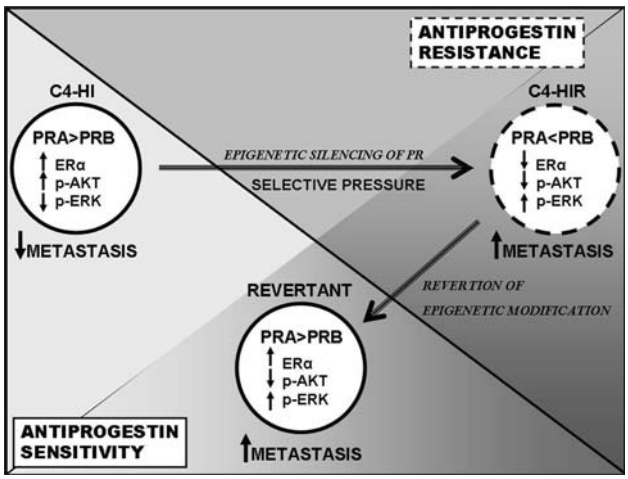


Fig. 8 Dissociation between hormone responsiveness, metastatic potential and kinase activation in MPA-induced mammary carcinomas. Sensitive tumors, which respond to antiprogestin treatment, express a high level of PR-A, low levels of pERK and high levels of pAKT. By comparison, tumors which acquire hormone resistance show lower levels of PR-A, higher metastatic ability and an inverse expression of kinases (high levels of pERK and low levels of pAKT). With time or after E_2 treatment, the resistant phenotype may be reversed. Reversion is accompanied by the reversion of the PR isoform phenotype. However, the metastatic phenotype or the kinase activation pattern is not reversed suggesting that epigenetic mechanisms may be downregulating hormone receptors and rendering the tumors insensitive to RU-486 treatment. In the process of selective pressure, a genotypic selection of more aggressive clones may favor tumor progression; however, this increase in aggressiveness is unidirectional, and could be not associated with the ability of tumors to respond to a hormone therapy

reversed by endocrine treatment. CpG Methylation of the ER has been one of the mechanisms proposed to explain a decrease in ER expression in some de novo-resistant tumors [47]. As PR can also be silenced by methylation [48], this might be an interesting possibility to explore. On the other hand, as the hormone reverted phenotype was not accompanied by a reversion of the metastatic phenotype, it may be suggested that the expression of PR A is unrelated to the metastatic phenotype. A scheme summarizing the transition to hormone resistance in our model is depicted in Fig. 8.

It is noteworthy that there are parallels between our results and those of others, indicating that resistant tumors have increased activation of the MAPK pathway, which in turn may favor phosphorylation of ERα [49, 50]. These studies have directly related the occurrence of increased growth factor receptor signaling to the hormone-resistant phenotype. Although activation of PR by MAPK has also been reported [51–53], our results suggest that AKT activation may be involved in the regulation of hormone receptor function in C4-HI tumors, whereas in C4-HIRev tumors, which respond similarly but are more metastatic than C4-HI tumors, the activation of MAPK may play a critical role in regulating hormone receptor function and/or tumor invasiveness.

There are several reports linking AKT activation to hormone resistance and even proposing AKT as a marker of hormone resistance [54]. However, it is possible that the regulation of kinases is a far more complex phenomenon than the expected, in which the different pathways may overlap in function depending on the cellular and tissue contexts. Our data showing more metastatic variants with decreased pAKT are in agreement with data reported by Toker and Yoeli-Lerner [55] indicating that the activated AKT1 isoform inhibits the transcriptional activity of NFAT, a downstream molecule directly related to the invasive phenotype. The decrease in pAKT may induce an upregulation of NFAT, which in turn might increase MMP-9 expression and other pathways involved in invasiveness and metastasis.

Finally, human breast carcinomas with a high PR-A/PR-B ratio are those which relapse earlier after TAM treatment [20], and in our breast cancer model, tumors with a high PR-A/PR-B ratio are those which respond to antiprogestin therapy. Taking these two facts into account, we propose that the subset of tumors with this PR expression pattern should be the target of antiprogestin therapies. In addition, as TAM has also been shown to increase PR expression, combined treatment of TAM or estrogens plus antiprogestins may be an alternative for acquired resistant tumors.

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