The MPA mouse breast cancer model: evidence for a role of progesterone receptors in breast cancer

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Abstract

More than 60% of all breast neoplasias are ductal carcinomas expressing estrogen (ER) and progesterone receptors (PR). By contrast, most of the spontaneous, chemically or mouse mammary tumor virus induced tumors, as well as tumors arising in genetically modified mice do not express hormone receptors. We developed a model of breast cancer in which the administration of medroxyprogesterone acetate to BALB/c female mice induces mammary ductal carcinomas with a mean latency of 52 weeks and an incidence of about 80%. These tumors are hormone-dependent (HD), metastatic, express both ER and PR, and are maintained by syngeneic transplants. The model has been further refined to include mammary carcinomas that evolve through different stages of hormone dependence, as well as several hormone-responsive cell lines. In this review, we describe the main features of this tumor model, highlighting the role of PR as a trigger of key signaling pathways mediating tumor growth. In addition, we discuss the relevance of this model in comparison with other presently used breast cancer models pointing out its advantages and limitations and how, this model may be suitable to unravel key questions in breast cancer.

Endocrine-Related Cancer (2009) 16 333–350

Introduction

Breast cancer is the most frequent cancer in women (23% of all cancers), and it ranks second overall when both sexes are considered together (Parkin *et al.* 2005). Most tumors are ductal infiltrating carcinomas expressing estrogen (ER) and progesterone receptors (PR). The majority of the genetically modified mouse breast cancer models as well as most spontaneous, chemically or mouse mammary tumor virus (MMTV)-induced mammary tumors in mice do not express ER and PR, or if they do (some MMTV models), they are pregnancy-dependent (Kordon 2008). One of the few exceptions is the medroxyprogesterone acetate (MPA)-breast cancer model. More than 20 years ago, we developed an experimental model in mice, in which MPA induced

mammary carcinomas that expressed high levels of ER and PR. The aim of this review is to assemble all the results of the last 20 years to better understand the possibilities and limitations of this model for the further understanding of breast cancer.

At the beginning of the 1980's, we became interested in the clinical observation that progestins may block growth in the benign but invasive fibroblastic proliferations known as desmoid tumors (Lanari *et al.* 1978, Lanari 1983). In trying to find a mouse model where we could reproduce these results, we decided to investigate the inhibitory effects of progestins on foreign body tumorigenesis in BALB/c mice. In this specific type of tumorigenesis, the subcutaneous (s.c.)

implantation of a glass cylinder in mice results in the formation of a thick fibrous capsule surrounding the cylinder. Within ~9 months, fibrosarcomas would develop from that capsule, with an incidence of 79% (Lanari *et al.* 1986b). To evaluate the effects of progestins on fibrosarcoma growth, we decided to use MPA rather than progesterone (Pg), because MPA compound was easier to handle, as it was available in a depot delivery form and it did not have to be injected on daily basis. We showed that in BALB/c mice treated with 40 mg MPA depot s.c., every 2–3 months (four doses), the number of foreign body fibrosarcomas that developed was significantly lower than in the untreated controls (Lanari *et al.* 1986b). Unexpectedly, the few MPA-treated female mice remaining at the end of the experiment developed mammary carcinomas. Two follow-up studies performed using exclusively female mice confirmed this carcinogenic effect, yielding multiple mammary carcinomas with a mean latency of 1 year and an average incidence of about 80% (Lanari *et al.* 1986a, Kordon *et al.* 1994). The fact that progestins could induce mammary neoplasias was rather counter-intuitive, as the consensus at that time (and for many years to come) was that, estrogens were the proliferative/carcinogenic hormones, whereas Pg exerted mainly differentiating effects, thus counter-acting the stimulatory properties of estrogens. However, there was already evidence that challenged this dogma. Pg or MPA administered in certain schedules of carcinogenesis protocols increased mammary tumor incidence in rats (Young 1961, Jabara 1967, Jabara *et al.* 1973), in mice carrying endogenous MMTV (van Nie 1964, Sluysers & Van Nie 1974), and in cats and dogs (Hernandez *et al.* 1975, Concannon *et al.* 1981). In addition, Pg (Watson *et al.* 1979) and MPA (Formelli *et al.* 1985) stimulated the growth of the MXT transplantable mouse tumors.

The carcinogenic effect reported for MPA was also supported by later studies in rats, cats, dogs, and mice (Russo *et al.* 1989, Misdorp 1991, Ohi & Yoshida 1992, Pazos *et al.* 1992, Aldaz *et al.* 1996, Goepfert *et al.* 2000, Benakanakere *et al.* 2006). The findings of the WHI Study (WHI 2002) and the Million Women Study (Beral 2003), showing increased breast cancer in estrogens-plus-progestin-treated women, further highlighted the risk associated with chronic progestin administration in humans. By 1999, a Working Group at the International Agency for Research on Cancer, World Health Organization (IARC) concluded that there was sufficient evidence in experimental animals for the carcinogenicity of MPA (limited evidence for carcinogenicity in dogs had been the previous evaluation in 1979; IARC Working Group 1999,

1999), and moved MPA to Group 2B (possibly carcinogenic to humans). In 2005, the IARC Working Group, on the basis of several studies that included postmenopausal therapy with estrogens plus MPA, considered that there was sufficient evidence in humans for carcinogenicity of combined estrogen–progestogen menopausal therapy in the breast, and moved it from Group 2B to Group 1 (carcinogenic to humans; IARC Working Group 2007). Various reports have also highlighted the proliferative role of progestins on breast cancer and have been recently reviewed (Moore 2004, Aupperlee *et al.* 2005, Kariagina *et al.* 2008).

MPA-induced carcinogenesis

Mammary carcinomas

In the first study, in which we reported the carcinogenic effects of MPA, the tumors were histologically classified as B adenocarcinomas according to Dunn's classification (Sass & Dunn 1979). As most of the neoplasias, as well as the preneoplastic lesions, were similar to the human ductal counterparts, we re-classified all tumors following the histological criteria used for the human disease. Accordingly, 68% of the tumors were ductal carcinomas and the other 32% were lobular carcinomas. Using a different protocol in which MPA was given as a s.c. 40 mg silastic pellet that was replaced by a 20 mg pellet 6 months later, MPA also proved to be carcinogenic, with an incidence of 58%, and, in this case, the ratio of ductal to lobular carcinomas was even higher, with 89% being ductal (Kordon *et al.* 1993). Ductal hyperplasias or carcinomas *in situ* were detected in most animals that did not develop carcinomas. In a few cases, lymph node and lung metastases were observed. Detached groups of tumor cells were also found within veins in different organs during histopathological evaluation of complete autopsies. All ductal mammary carcinomas expressed high levels of ER and PR, as well as prolactin receptors as evaluated by ligand binding techniques. Prolactin and epidermal growth factor (EGF) receptors were detectable in purified membrane fractions (Molinolo *et al.* 1987, Lanari *et al.* 1989). The low percentage of lobular carcinomas arising from MPA-treated animals, were very similar in morphology to those originating in other experimental models (IARC 1994, Seely & Boorman 1999, Greaves 2007), as well as to spontaneous carcinomas occasionally found in aging, multiparous BALB/c and other mice strains (Rehm & Liebelt 1996 and personal observation). Similarly, to what happens in these models, the lobular tumors may

express when evaluated by binding techniques, low levels of ER and PR. However, and unlike what happens with the ductal carcinomas, the expression of ER and PR is lost, if transplanted subcutaneously, after a few passages (Kordon *et al.* 1993, 1994). The nomenclature ‘lobular’ was given to these tumors because their preneoplastic lesions mimicked those of human lobular carcinomas, which are characterized by an increase in the number of alveolar structures with progressive enlargement due to intra-alveolar growth, as well as because they would occasionally infiltrate the stroma in linear arrays of malignant cells, the ‘Indian files’ images also seen in human lobular cancer, but it is quite possible that they represent a different biological entity.

Other effects of MPA

MPA-treatment was also associated with the early development of endometrial glandular cystic hyperplasias and/or deciduomas regardless of the presence of mammary carcinomas (Lanari *et al.* 1986a, Molinolo *et al.* 1987). MPA also induced the differentiation of the granular convoluted tubules in the female’s submaxillary salivary glands (Kordon *et al.* 1994, Montero Girard *et al.* 2007), leading to an increase in gland size. This had already been shown by Bullock *et al.* (1975) as part of the androgenic effect of MPA. The hypertrophy was associated with an increase in EGF synthesis and an increase in serum EGF levels. BALB/c or C57BL/6 mice treated with MPA increased their body weight (Pazos *et al.* 1998, Montero Girard *et al.* 2007). The administration of MPA to mice, 7 or 90 days before immunization with sheep red blood cells, significantly enhanced both primary and secondary antibody responses, without affecting delayed-type hypersensitivity (Vermeulen *et al.* 2001). Additionally, we demonstrated that MPA decreased the incidence of leukemias, while it did not affect the incidence of lung adenomas in *N*-methyl-*N*-nitrosourea (MNU)-treated mice (Pazos *et al.* 2001).

MPA versus progesterone

Pg administration also induced mammary carcinomas when given as 40 mg pellets replaced by 20 mg pellets after 6 months, although the incidence was lower than that obtained with MPA (28 vs 58%; Kordon *et al.* 1993). Only 28.5% of the Pg-induced carcinomas were ductal; while the rest showed lobular differentiation, according to the histological criteria mentioned above. In the uterus, Pg induced a microglandular hyperplasia; cystic lesions as well as deciduomas were consistently absent.

Correlation between histology and hormone dependence

To evaluate hormone dependence (HD), MPA or Pg-induced carcinomas were transplanted into MPA-treated or untreated mice. The tumors that did not grow during the first 2 months in untreated animals were considered HD, or more specifically progesterin-dependent (PD), while those that did grow were designated hormone-independent (HI), or progesterin-independent (PI). Out of 48 carcinomas in which histology and HD were recorded, 15 were lobular and HI, 32 were ductal and HD, and only one ductal tumor was HI. Histological evaluation was performed blinded to the HD status, and it proved to be a good predictor of HD in this model (Kordon *et al.* 1993, 1994).

Strain specificity

To evaluate the strain-specificity for the carcinogenic effect of MPA, we tested C3H and C57BL/6 female mice using the standard protocol of MPA depot. The incidence of mammary carcinomas was significantly different between MPA-treated C3H mice and untreated controls (32 vs 16%, $P < 0.05$). C57BL/6 MPA-treated female mice did not develop mammary carcinomas. Moreover, MPA and Pg induced morphological changes in the mammary glands of C57BL/6 different from those seen in BALB/c mice (Montero Girard *et al.* 2007). Similar results have recently been shown by Aupperlee *et al.* (2009). Along this line, we also demonstrated that the expression of ER α and of the A isoform of the PR (PR-A) was lower in virgin C57BL/6 mice than in BALB/c mice. Interestingly, when epithelial mammary cells from both strains were transplanted into cleared mammary fat pads of the same immunocompromised mouse, both morphological and receptor expression differences were abolished, thus reinforcing the role of the microenvironment in mediating epithelial hormone responsiveness. These results highlight the role of the altered expression of susceptibility/resistance genes in both, anomalous hormone responsiveness and breast cancer.

Effects of sialoadenectomy or ovariectomy in MPA-induced carcinogenesis

The initial observation that female mice treated with MPA developed salivary glands hypertrophy led us to look for factors derived from such glands that may have played a role in mammary carcinogenesis. As previously stated, MPA induces through androgenic actions, the differentiation of convoluted granular

tubules that are responsible for the synthesis and secretion of several growth factors and EGF among them (Bullock *et al.* 1975). We showed that in BALB/c mice sialoadenectomy (Sx) lowered the incidence of MPA-induced mammary carcinomas and that this was associated with diminished branching of the mammary glands (Molinolo *et al.* 1996). Inoculation with EGF was able to restore and even increase branching (Molinolo *et al.* 1998). Sx also affected the induction of MNU-induced carcinomas in MPA-treated mice (Molinolo *et al.* 1996). In the rat, EGF has also been demonstrated to participate in MNU-induced mammary carcinogenesis (Chou *et al.* 1999).

In ovariectomized (ovx) mice, the incidence of MPA-induced mammary carcinomas was significantly lower than in sham-operated animals, and the same was true for MNU+MPA-induced mammary tumors (unpublished observations, E Kordon, P Pazos, C Lanari & AA Molinolo). In ovx mice, the lack of estrogen levels high enough to induce the expression of physiologically relevant quantities of PR in the mammary gland, may explain the lesser carcinogenicity of MPA. The fact that some tumors did develop, in spite of the absence of a significant estrogenicity, may be explained by the fact that MPA induces the synthesis and secretion of salivary glands' EGF, a factor that has been shown to exert estrogenic effects (Bunone *et al.* 1996).

MPA-induced mammary carcinomas: tumor transplants

Hormone dependence

Progestin-induced mammary carcinomas were subcutaneously transplanted into the inguinal flank of female BALB/c mice treated or non-treated with MPA. If the tumors grew only during the first 2 months in treated mice, they were considered PD or HD.

HD growth

Ovariectomy delayed tumor growth in both MPA-treated and in untreated animals. In untreated ovx mice, the tumors grew very slowly after more than 6 months, which was probably due to adrenal hormones, since no growth was observed in ovx/adrenalectomized animals (Kordon *et al.* 1990, Montecchia *et al.* 1999a).

When tumors were transplanted into ovx animals, Pg was able to stimulate HD growth nearly as well as MPA, while dihydrotestosterone (DHT) exerted a slight proliferative effect (Kordon *et al.* 1990). It has been demonstrated that the administration of 8-Cl-cAMP (Actis *et al.* 1995), TNF α (Rivas *et al.* 2008) or fibroblast growth factor (FGF-2) (Giulianelli

et al. 2008) can also mimic the MPA effect *in vivo*. Although, most of the HD tumors studied showed similar patterns of hormone dependence, the degree of this dependency varied among HD tumors. The tumors with the highest degree of hormone dependence would stop growing and even regress after hormone withdrawal (Simian *et al.* 2006), while others would stop growing or continue to grow very slowly. When transplanted into intact immunocompromised mice, they showed the same MPA requirement (Kordon *et al.* 1994).

HI growth

Occasionally, these HD tumors start to grow in untreated animals (Fig. 1). At this point, we suggest that this tumor has become HI. When this happens, the original HD tumor is recovered from frozen samples from early passages, while the HI tumor is maintained by syngeneic transplantation in untreated BALB/c mice. These HI tumors grow in both intact and ovx mice and some of them grow faster in non-ovx animals.

Treatment responsiveness and tumor regression

The administration of 17 β -estradiol (E₂; 5 or 0.5 mg silastic pellets) exerted a clear inhibitory effect in s.c. implanted tumors and in primary cell cultures (Lamb *et al.* 2003). It is interesting to note, as previously mentioned that ovx in these animals is associated with a significant reduction of MPA carcinogenicity. It seems that physiological estrogen levels are required to induce PR expression in the mammary gland, which in turn may be necessary for tumor induction. However, in the established tumors, the sustained pharmacological serum concentrations achieved through the external E₂ administration results in a potent inhibitory signal.

Interestingly, and as opposed to what happens in most of the traditional mouse models, pregnancy inhibits R-PI tumor growth (Bustuoabad *et al.* 2002). It is possible that the increased E₂ levels observed in pregnancy might be counteracting the proliferative effect of Pg. The administration of three different antiprogestins, mifepristone (RU-486), onapristone (ZK 98299) or ZK 230211 (Hoffmann & Sommer 2005), in daily s.c. doses of 6–12 mg/kg body weight, also inhibited tumor growth or induced complete tumor regressions in most of the ductal HI variants growing in BALB/c (Kordon *et al.* 1991, Montecchia *et al.* 1999a, Vanzulli *et al.* 2002, Wargon *et al.* 2008) or nude mice (unpublished observations, R Soldati, A Vanzulli, AA Molinolo & C Lanari). The *in vivo* administration of PR antisense oligodeoxynucleotides (asPR)

Figure 1 Tumor transplants and generation of tumors. The continuous administration of MPA to female BALB/c mice induces HD mammary carcinomas, which are maintained by syngeneic transplants into MPA-treated mice. Two mice are left untreated to control HD. Occasionally, tumors start to grow in untreated mice and, as a result, a HI tumor is established. These tumors grow even in the absence of MPA. Some of those HI tumors are inhibited by the treatment with antiprogestins (RU486), estrogens (E₂) or tamoxifen (TAM; responsive HI: R-PI), and other HI tumors are unresponsive to the hormonal treatment (unresponsive HI tumors: UR-PI).

induced a transient inhibition of tumor growth supporting the key role of PR in tumor growth (Lamb *et al.* 2005b). The antiandrogens hydroxyflutamide and flutamide had no effect (Montecchia *et al.* 1999a).

Since some HI or PI variants did not respond to estrogens or antiprogestins, we had to re-classify PI tumors as responsive (R-PI) and unresponsive (UR-PI) tumors (Helguero *et al.* 2003b; Table 1). The four UR-PI tumor variants may be considered as *de novo*

resistant tumors and were resistant to both estrogens and antiprogestins (Figs 1 and 2). The specificity of the RU486 and MPA effects was assayed using different transplantable tumor models, such as a syngeneic lymphoma, LB, and a methylcholantrene-induced fibrosarcoma (Bustuoabad *et al.* 2002), and no significant differences between treated mice and controls were observed (unpublished observations, C Lamb, V Fabris, C Lanari & AA Molinolo).

Tamoxifen (daily s.c. injections of 5 mg/kg body weight) induced an inhibition of growth in R-PI and in HD tumors growing either in the presence or in the absence of MPA (Lamb *et al.* 2003). In this model, tamoxifen behaves as an estrogenic agonist, albeit with lower efficacy than estrogens. Raloxifene was only assayed in HD tumors; it had no effects in tumor growth *in vivo* (12.5 mg/kg daily doses). Doxorubicin, administered as pegylated liposomes (Doxopeg, Laboratorios Raffo, Argentina; 9 or 18 mg/kg once a week), induced significant growth inhibition (unpublished observations, R Soldati, I Luthy, L Colombo, AA Molinolo & C Lanari).

The effect of $\alpha(2)$ -adrenoceptor agonists and antagonists has also been studied in our model, as stress may be an important modulator of breast

Table 1 MPA-induced mammary carcinomas

Hormone dependent	Hormone independent		
	Responsive	Unresponsive	
		Acquired	De novo
C4-HD	C4-HI	C4-HIR ^a	C4-2-HI
↓	CC4-HI		
CC4-HD ^b	CC4-3-HI		
C7-HD	C7-2-HI ^a	BET	C7-HI ^a
32-HD	32-2-HI		32-HI
48-HD	48-HI		
59-HD	59-2-HI	59-2-HIR	59-HI

^aHighly metastatic in axillary lymph nodes and lungs.

^bPrimary cultures of C4-HD were inoculated subcutaneously and maintained by serial transplantation.

Figure 2 Working hypothesis. PR play a key role regulating tumor growth. In HD (PD) carcinomas, MPA or Pg interact with PR inducing a proliferative state characterized by the presence of high levels of stimulating growth factors such as IGF-II and HRG and low levels of inhibitory factors such as TGFβs. If tumors are able to grow without exogenous hormone supply they are considered HI or PI. In these tumors, we have hypothesized that paracrine stromal factors mimic Pg signaling, and therefore, the tumors have a growth pattern similar to PD tumors growing with MPA. FGF-2 is one of the stromal paracrine growth factors inducing PR activation through binding with FGFR-2, its cognate receptor, in the epithelial cells. Therefore, the blockage of PR will induce effects similar to progesterone removal in the PD tumors: cytostasis and apoptosis. These tumors are responsive progesterone-independent tumors (R-PI) and, as PD tumors, they have higher expression of PR-A than PR-B. However, in some PI tumors, PR inhibition with antiprogestins does not inhibit tumor growth (UR-PI). These tumors still express PR but they have a different pattern of isoform expression, as observed by western blots: higher expression of PR-B than PR-A.

cancer growth. Clonidine significantly enhanced tumor growth while the antagonists yohimbine and rauwolscine, completely reversed the effects of clonidine. Rauwolscine alone diminished tumor growth significantly, behaving as a reverse agonist (Bruzzone *et al.* 2008).

Cytostasis, apoptosis (Vanzulli *et al.* 2002) or differentiation (Wargon *et al.* 2008) are the hallmarks of tumor regression. The morphological changes of tissue remodeling were preceded by an early increase in p53, p21, and p27 expression. ER and PR expression were down-regulated 48 h after the onset of tumor regression (Vanzulli *et al.* 2002, 2005). Concomitantly, with the increase in apoptosis, there was also an increase in tissue remodeling (Simian *et al.* 2006). Interestingly, an increased expression of the CDK inhibitors p21 and p27 was also observed in primary cultures of C4-HD cells treated with TGFβ1 (Salatino *et al.* 2001), suggesting that antiprogestins and estrogens may increase TGFβ activity. In T47D human breast cancer cells, antiprogestins have also been shown to induced an increase in TGFβ1 expression (Dannecker *et al.* 1996). The chronology of events that lead to tumor regression, as well as the mechanisms by which E₂ and antiprogestins converge

to induce tumor regression, are now actively being studied in our laboratory.

Estrogen and progesterone receptors

Two different binding sites were observed for PR, one with a high capacity and with an affinity similar to the standard K_d described for PR (K_d : 9.2 nM; $Q=376$ fmol/mg protein; Bayard *et al.* 1977), and a second low-capacity, high-affinity binding site with a K_d of 43 pM (Helguero *et al.* 2003a). ER were also detected by binding techniques with a standard K_d of 1.5 nM.

All the studied ductal tumors expressed ERα and ERβ as determined by western blotting. Both PR-A (83 kDa) and PR-B (115 kDa) were detected in HD and R-PI tumors. PR-A expression was always higher than PR-B (Helguero *et al.* 2003b, Wargon *et al.* 2008) and MPA down-regulated both PR isoforms (Helguero *et al.* 2003b). In UR-PI tumor samples, PR-A expression was almost undetectable (Helguero *et al.* 2003b, Wargon *et al.* 2008). In addition, RNase protection assays did not reveal any differences in total mRNA between R-PI and UR-PI tumors. The western blot data were corroborated by immunohistochemistry, using antibodies specific for PR-B and PR-A

(Aupperlee *et al.* 2005, Wargon *et al.* 2008). In addition, in immunofluorescence studies, we observed that both PR isoforms were co-expressed in the same cells.

The UR-PI tumors may be considered as *de novo* or constitutively resistant tumors. Using selective pressure, we have also generated estrogen- and antiprogesterin-resistant tumors. We have shown that acquired estrogen resistance (Montecchia *et al.* 1999b) or acquired antiprogesterin resistance (Wargon *et al.* 2008), are reversible phenomena. These acquired antiprogesterin-resistant variants had, as *de novo* resistant tumors, lower levels of PR-A than PR-B, suggesting that the PR isoform ratio is predictive of antiprogesterin responsiveness (Wargon *et al.* 2008).

Growth factor receptors and ligands

Similar levels of insulin-like growth factor 1 (IGF-I) and IGF-1 receptors, detected by RNase protection and by radio receptor assays, were found in HI and HD tumors in the presence or absence of MPA. IGF-II, on the other hand, was up-regulated by MPA and was highly expressed in HI tumors (Elizalde *et al.* 1998). Man 6P/type II IGF receptors were down-regulated by MPA. The blockage of IGF-1 receptors by antisense oligodeoxynucleotides *in vivo* induced a delay in tumor growth that does not seem to be mediated by PR (Salatino *et al.* 2004), suggesting that IGF-I signaling is downstream of PR.

The expression of heregulin (HRG), c-erbB2, and c-erbB3 was up-regulated by MPA in HD tumors, reaching levels similar to those observed in the HI tumor variants. c-erbB4 expression that was not regulated by MPA, was similar in HD and HI tumors (Balana *et al.* 1999, 2001). TGF β s 1, 2, and 3 were down-regulated by MPA in HD tumors, and their expression levels were lower in the HI variants. TGF β 1R and 2 were present in HD ductal tumors, but only TGF β 1R was significantly detected in the HI variants (Elizalde *et al.* 1995, Viegas *et al.* 1999). FGFR-2 was also highly expressed in HI tumors and up-regulated by MPA in the HD tumors (Giulianelli *et al.* 2008).

All these results indicate that R-PI tumor variants are biologically similar to their respective parental tumors, reinforcing the hypothesis that in R-PI tumors, PR are activated by signals that mimic the progestin effect. We hypothesize that the increase in growth stimulatory factors and the decrease in inhibitory factors are intrinsic to the proliferative state of the tumors, and that they may act in concert downstream of PR, triggering mitotic and antiapoptotic signals (Fig. 2).

Trp53 mutations and cytogenetic analysis

Five different HD tumors as well as some of their derived HI variants, and two lobular tumors were studied by PCR-SSCP and sequenced for *Trp53* mutations. C4-HD, and most of its HI variants had the same point mutation, a C–G change at position 456 in exon 5, representing a serine to arginine mutation at codon 152, within the DNA binding site (S152R; Fabris *et al.* 2005). p53 is one of the most frequently mutated proteins in human cancer (Bourdon 2007). This transcription factor is a key regulator of cell cycle and apoptosis, which is activated in response to different stresses, genotoxic as well as not genotoxic, and modulates the transcription of several genes (p21, mdm2, GDD45, bax). p53 is also involved in DNA repair and centrosome stability. The untimely activation, inactivation or otherwise deregulation of any of these processes could arguably contribute to the development and maintenance of the malignant phenotype. Between 20 and 35% of human breast tumors present in mutations p53, occurring principally on the DNA binding domain (Lacroix *et al.* 2006).

The functionality of the C4-HD *p53* mutations was assessed in studies using its derived MC4 cell lines, which carry the same mutations. The exposure of these cells to UVB does not induce an increase in p21.

A nearly diploid chromosome number ($2n=40$) was found in three of the five HD tumors, while numbers in the triploid to tetraploid range were observed in the other two HD tumors (Fabris *et al.* 2005). Some HI tumors were diploid, while most of them were aneuploid (8/12 tumors). The most frequent alterations found in HD and HI tumors were gains of chromosomes 3, 4, and 6, and losses of chromosomes 16 and X. Chromosomes 4 and 7 were involved in translocations in three of the four tumor families studied. We evaluated the evolution of the karyotype in the transition to HI and have demonstrated that HI may be acquired without changes in ploidy, suggesting that the increase in ploidy observed in many tumors is favored by successive transplantation. In our model, all diploid tumors responded to hormone treatment (E_2 or antiprogesterins), while aneuploid tumors were either responsive or not (Fabris *et al.* 2005).

Mutations in the *p53* gene are frequently associated to aneuploidy and chromosome instability. However, the fact that C4-HD tumor, unlike its HI counterparts, maintains a rather stable karyotype during *in vivo* passages may be indicating that other factors in concert with p53 may drive chromosome instability.

Metastatic ability

All ductal carcinomas assayed gave rise to metastases in lymph nodes and lungs. Some of these tumors are specially suited for this type of studies as they develop metastases early, usually within 2 months of transplantation (Table 1). In general, lymph node metastases tend to be histologically more differentiated than the s.c. implant, a fact that is apparently unrelated to the selection of a specific cell subpopulation. When lymph nodes metastases are s.c. transplanted into syngeneic animals, the histology reverts to that of the originally primary tumor, displaying now the less differentiated phenotype, a fact that underscores once again the significant role of the microenvironment in regulating the specific tumor features. These results are in agreement with recent findings showing that in humans, tumor cells in lymph node metastases have a CD24+, luminal phenotype (Shipitsin et al. 2007). Hormone receptors are still expressed in both lymph node and lung metastases (Vanzulli et al. 2005), at levels similar to those of the primary tumor, and antiprogestins and estrogens induced regression of the metastasis. This phenomenon is associated with increased cell differentiation, increased expression of p21 and p27, and down-regulation of ER and PR (Vanzulli et al. 2005). It is worth pointing out that this is one of the very few models of lymph nodes metastases in murine mammary carcinomas (Vargo-Gogola & Rosen 2007).

In vitro studies

Primary cultures

To obtain epithelial or fibroblastic enriched cultures, we use standard protocols (Pandis et al. 1992), with slight modifications (Dran et al. 1995). To test the effects of steroids or growth factors, cells are always grown with DMEM F12-HAM in the presence of 1–5% steroid-stripped serum.

Epithelial cells

MPA- and Pg-stimulated cell proliferation at concentrations as low as 10^{-12} M. DHT had no effect and dexamethasone was stimulatory at concentrations higher than 10 nM (Dran et al. 1995). E_2 exerted an inhibitory effect even at low concentrations (Dran et al. 1995, Lamb et al. 2003). The anti-progestins, RU486, and ZK 98299 (Lamb et al. 1999), exerted a striking inhibitory effect, while the antiandrogen hydroxyflutamide had no effect. The inhibitory effect of RU486 was observed at concentrations as low as 10 nM. It has been reported that 10 nM RU486 does not bind to the

glucocorticoid receptors in T47Dco cells (Horwitz 1985), thus supporting the role of PR in mediating cell growth. All effects were specific for C4-HD, since no changes were observed in primary cultures from a MPA-induced lobular carcinoma or in a spontaneous tumor that arose in a multiparous BALB/c mouse (Dran et al. 1995). The anti-estrogen fulvestrant (ICI 182780) as well as the selective estrogen receptor modulators, tamoxifen, and raloxifene, all inhibited cell proliferation. In the presence of MPA, fulvestrant and tamoxifen were inhibitory and raloxifene stimulated MPA-induced cell proliferation. As for the growth factors, EGF, IGF-I or IGF-II, they exerted almost no stimulatory effect when administered alone (Elizalde et al. 1998, Molinolo et al. 1998); however, IGF-I increased MPA-induced cell proliferation. On the other hand, FGFs and HRG exerted a stimulatory effect on MPA-treated or -untreated cells (Lanari et al. 1997, Balana et al. 1999, Lamb et al. 1999). TGF β 1–3 were all inhibitory (Viegas et al. 1999). Interestingly, serum from MPA-treated ovx mice stimulated cell proliferation to levels higher than that from ovx mice, to which MPA was added exogenously, indicating that other serum factors in addition to MPA are participating in tumor growth (Lamb et al. 1999). These results are in agreement with data reported in T47D-YB cells in which progestins, in addition to inducing cell proliferation, also stimulate the production of soluble factors that would be responsible for the sustained MAPK signaling leading to cell growth (Faivre & Lange 2007).

Fibroblasts

Carcinoma-associated fibroblasts (CAFs) were unresponsive to progestins, estrogens or androgens. Anti-hormones at concentrations of 10^{-6} M induced minor inhibitory effects, except for RU486, which slightly stimulated cell proliferation. On the other hand, all growth factors tested, including the TGF β s, were stimulatory for fibroblasts (Lanari et al. 1997). CAF from HI tumors expressed higher levels of FGF-2 and hepatocyte growth factor than CAFs from HD tumors (Giulianelli et al. 2008), leading to the idea that CAFs participate in HI growth (Fig. 2).

Cell lines

We have developed several cell lines from C4-HD (Lanari et al. 2001) and C7-2-HI tumors (Efeyan et al. 2004). The most interesting feature of these cell lines is that, unlike most murine mammary cancer cell lines, they retain ER and PR expression, although at levels lower than those of the primary cultures from the same

tumors (Lanari *et al.* 2001, Aliaga *et al.* 2004). These cell lines showed different degrees of hormone responsiveness *in vitro*, and both estrogens and progestins were stimulatory. Anti-progestins stimulated cell growth at low concentrations, but they inhibited cell proliferation at concentrations higher than 100 nM. Estrogens, unlike what happens in the parental tumors (both *in vivo* and in primary cultures), where they behave as inhibitory agents, may stimulate cell proliferation. Even though preliminary data of our laboratory suggest that this difference is not related to the expression of specific ER α or ER β isoforms, we cannot rule out a role for the ER α splice variant ER 36 (Wang *et al.* 2006). This variant has been demonstrated to be involved in cell proliferation, and the fact that it may be active only in the cell lines remains to be explored. The complete absence of the modulating influence of stromal ECM in the cell lines, as opposed to what happens in transplants may also help to explain the differential estrogen response. Others have reported differences in hormone responsiveness between *in vitro* and *in vivo* settings; MCF-7 cells are stimulated *in vitro* by estrogens, whereas *in vivo* the presence of the hormone is an absolute requirement, as they simply do not grow without estrogen supplementation in immunocompromised animals (Shafie 1980). The reason of this difference remains still unknown.

Two cell lines, a cloned epithelial tumor cell line, MC4-L4E, and a stromal non-tumorigenic cell line, named MC4-L4F, were also developed from the parental tumor C4-HD. Both express ER α and low levels of PR, show increased epithelial cell growth and an increase in PR expression when co-cultured under starvation conditions. These characteristics provide an interesting tool to study stromal–parenchymal interactions (Lamb *et al.* 2005a).

PR, key player mediating cell proliferation

In 1993, we found that RU486 was able to completely inhibit MPA-induced and steroid stripped serum-induced cell proliferation in HD cells, suggesting that PRs were playing a pivotal role in mediating this effect. At that time, the concepts of ‘ligand-independent activation’ and ‘crosstalk’ were gaining acceptance and Edwards *et al.* (1993) had suggested that PR could be activated by cAMP analogues. FGFs were the only ones of a series of growth factors tested that stimulated cell proliferation like MPA (Lamb *et al.* 1999). Using different anti-progestins and asPR, we demonstrated that FGFs stimulated cell proliferation via the PR pathway (Lanari *et al.* 1997). Later, Labriola *et al.* (2003) used the same cells and HRG instead of

FGFs to confirm a crosstalk between MAPK and PR in the MPA tumor model.

All the results obtained using the HD tumors suggested that HI tumors might have ligand-independent activated PR (Montecchia *et al.* 1999a, Lamb *et al.* 2005b). Anti-progestins induced complete tumor regressions and asPR (1 mg every 12 h for 5 days) were also inhibitory. We measured the Pg concentration in both HD and HI tumors and found no differences (0.1–0.5 ng/ml; RIA). The remaining unanswered question was what activated PR in HI tumors. Our working hypothesis, as depicted in Fig. 2, was that PD tumors were progestin- and PR-dependent while R-PI tumors were only PR-dependent. We hypothesized that stromal factors, such as FGF-2, might be responsible for activating PR. The fact that (a) isolated epithelial cells from C4-HI cultures were as MPA- or FGF-2-dependent as epithelial cells from C4-HD cultures and (b) CAF from HI tumors were much more stimulatory than CAF from HD tumors, led us to propose that factor/s secreted by CAF from HI tumors may be participating in the HI phenotype and FGF-2 was a likely candidate. Indeed, FGF-2 does activate PR in C4-HI cells; a neutralizing FGF-2 antibody and the genetic or pharmacological blockade of FGFR-2 inhibit CAF-induced epithelial cell proliferation and PR activation (Giulianelli *et al.* 2008). This *in vitro* data, together with the *in vivo* data demonstrates that FGF-2 stimulated C4-HD growth and that the FGFR inhibitor (PD 173074) decreased HI growth, which underscores the role of CAF in HI tumor growth.

Estrogen and antiprogestin in breast cancer treatment

Estrogens were extensively used for the treatment of breast cancer prior to tamoxifen (Carter *et al.* 1977) and have been experimentally shown to induce tumor regression in the T61 human breast tumor transplant model in nude mice (Brunner *et al.* 1996). Cell lines overexpressing PKC α (Chisamore *et al.* 2001, Lin *et al.* 2006) and cells which become addicted to tamoxifen (Yao *et al.* 2000) are inhibited by estrogens. The possibility of exploiting these findings in breast cancer treatment has been recently reviewed (Jordan 2008).

Antiprogestins, on the other hand, have been shown to inhibit experimental mammary cancer. In breast cancer patients, positive responses with RU486 have been reported (Klijn *et al.* 1989). In this series, most side effects were related to the antiglucocorticoid activity of RU486, thus illustrating the need of pure

antiprogestins (Klijn *et al.* 2000). Both RU486 and ZK 98299 were also shown to inhibit DMBA and MNU-induced mammary carcinomas in rats, as well as the MXT mouse tumors (Michna *et al.* 1989a,b, Schneider *et al.* 1989). Apoptosis and tumor differentiation were the mechanisms related with antiprogesterin-induced tumor regression (Michna *et al.* 1992a,b, Vollmer *et al.* 1992). Differentiation was also induced in normal mammary glands (Li *et al.* 1995). The T61 human xenograft model, inhibited with estrogens as mentioned above, was also inhibited by ZK 98299, although in this case E₂ priming was necessary to increase the expression of PR (Schneider *et al.* 1992). The authors hypothesized that the inhibitory effect of ZK 98299 should involve other mechanisms in addition to its antiprogesterin activity. In our model, antiprogestins only induced significant growth inhibition in PR-positive tumors. In other models, using s.c. transplantable tumors, only slight differences were seen (Check *et al.* 2007). It is possible, as NK have been shown to be modulated by progestins (Arruvito *et al.* 2008), that an immune-mediated mechanism may contribute to their efficacy (Check *et al.* 2007). Several groups have reported that the combination of antiprogestins and antiestrogens in breast cancer treatment was more efficacious than single drug treatments (Klijn *et al.* 2000). Experimentally, in MCF-7 cells transplanted in estrogen-treated nude mice, the simultaneous administration of tamoxifen and antiprogestins induced complete tumor inhibition. Monotherapy with tamoxifen or antiprogestins induced only a retardation of growth (el Etreby & Liang 1998, el Etreby *et al.* 1998). Similarly, the Pg-induced stimulation of T47D xenotransplants was suppressed by RU486 (Liang *et al.* 2007). These authors highlighted the proangiogenic role of MPA in breast cancer growth (Liang & Hyder 2005). Noteworthy, Poole *et al.* (2006) have recently reported that RU486 administration to BRCA1/p53-null female mice inhibits the generation of DMBA-induced tumors, as well as mammary gland branching.

A series of breast cancer clinical trials with different antiprogestins have already been reviewed (Klijn *et al.* 1994, 1996, Shi *et al.* 1994). Even though ZK 98299 showed less antiglucocorticoid activity than RU486, it had to be withdrawn from the market because of liver toxicity (Klijn *et al.* 2000). Interestingly, when PR-B is activated by high levels of cAMP, RU486 acts as an agonist (Horwitz 1992, Baulieu 1997), thus stimulating cell proliferation. Because of this, there has been certain resistance to the use of these agents for breast cancer treatment. The novel antiprogesterin ZK 230211 has lower antiglucocorticoid activity, and it does not

induce agonistic effects in the presence of protein kinase A activators (Fuhrmann *et al.* 2000). Accrual is now ongoing for breast cancer patients for a phase II clinical trial (<http://clinicaltrials.gov/ct2/show/NCT00555919>) using this drug. An even newer development is CDB-4124 and its putative metabolite CDB-2914, an antiprogesterin with almost no antiglucocorticoid effects (Attardi *et al.* 2002, 2004, Wiehle *et al.* 2007).

MPA-induced mammary carcinomas versus human breast cancer and other breast cancer models

Present classifications of human breast cancers are based on their specific molecular profiles, rather than exclusively on their histological features (Hu *et al.* 2006). The PI or HI tumors of the MPA breast cancer model share many features with the luminal breast cancers: a) most tumors are of ductal histology; b) they are invasive and metastatic; c) they are hormone responsive; d) express ER and PR; and e) respond to chemotherapy (Table 2). Some of them respond to estrogens, tamoxifen, and antiprogestins. Similarly, some human breast cancers are responsive to tamoxifen and to estrogens. Interestingly, 30% of established human breast cancer cell lines are ER-positive and they are stimulated by E₂. Several cell lines derived from the murine tumors of this model are also stimulated by E₂.

Mouse breast cancer models expressing steroid receptors

The MXT carcinoma (Watson *et al.* 1979) was one of the earliest ER(+), PR(+)-described tumors. This tumor model has been used for evaluating of the effects of chemotherapeutic agents and different hormones, as well as to test the effects of antiprogestins (Darro *et al.* 2005). Other strains such as GR or BALB/c, carrying the MMTV, develop pregnancy-dependent tumors that regress after parturition (Kordon 2008). Tumors of these models have a pattern of hormone-responsiveness completely different than most of the human breast carcinomas and they have been useful to study MMTV-activated oncogenes (Kordon 2008). The M05 BALB/c mouse tumor (Simian *et al.* 2008) arising spontaneously in a virgin female mouse, looks promising for hormonal studies, as tumor growth is inhibited with tamoxifen. The fact that this is the only one tumor, rather than a set of neoplastic proliferations, limits data validation.

Table 2 Comparison between MPA-induced mammary carcinomas and human breast cancer

MPA-induced mammary carcinomas	Human breast cancer
Ductal carcinomas	78% are ductal carcinomas; Glass et al. (2007)
Metastasis in lymph nodes and lung	Metastasis in lymph nodes and bone
ER(+), PR(+)	70% are ER(+), PR(+)
c-erbB2 (+)	20–25% are c-erbB2(+)
p53 mutations in 20% of the tumors	p53 mutations in 20% of the tumors
Responsive to endocrine treatment	Responsive to endocrine treatment
Clinical progression to hormone resistance	Clinical progression to hormone resistance
May regress completely by estrogen treatment	Estrogens have been successfully used for treatment in a group of patients
May be responsive to tamoxifen treatment	Different degrees of tamoxifen sensitivity
May regress completely by antiprogesterin treatment	There has been some responsiveness to antiprogesterin treatment in few trials
Responsive to doxorubicin	Responsive to doxorubicin
Cell lines are stimulated by E ₂	30% of the derived cell lines are stimulated by E ₂
Progestins may stimulate tumor growth	Progestins may inhibit or stimulate tumor growth

Xenografts in immunodeficient animals

These are the most common models used to evaluate hormone-related breast cancer and most of the studies come from a rather limited set of cell lines inoculated in nude or SCID mice, including MCF-7, T47D, ZR-75. None of these cell lines originate metastasis *in vivo* unless genetically modified, except for the recently developed IBH-4, IBH-6, and IBH-7 ([Bruzzone et al. 2009](#)). The fact that the human cells are growing in an immunodeficient mouse environment constitute obvious shortcomings of these approaches. New models have been developed in which human fibroblasts were transplanted together with the neoplastic cells, thus creating a humanized environment, although the possible role of the immune system has not been yet properly addressed ([Proia & Kuperwasser 2006](#)).

Genetic engineered mouse models

As recently reviewed ([Vargo-Gogola & Rosen 2007](#), [Allred & Medina 2008](#)), there are no mouse engineered models in which the induced mammary tumors give rise to metastasis either in lymph node, brain or bone. In addition, there are no consistent reports on metastatic carcinomas responding to hormone therapy, even though models are available in which hyperplastic growths retain ER and PR expression, such as a fraction of tumors arising in mammary glands from p53 null mice transplanted into in a BALB/c background. In other models, epithelial cells are genetically manipulated to express ER α , transformed *in vitro* and then transplanted into mice, resulting in metastatic estrogen-responsive tumors ([Duss et al. 2007](#)).

Summary and future directions

Our studies underscore the carcinogenic and proliferative effects of MPA in the BALB/c mammary gland. They also indicate that the effects of MPA on the mammary gland are different from those of Pg. Both compounds have different carcinogenic effects, although both substances are able to stimulate the growth of already established tumors. Using this model, we have also given a hierarchical role to the PR, as mediators of tumor growth. Progesterin-independent growth was inhibited by antiprogesterins and asPR suggesting that these tumors, although progesterin independent, were still PR dependent for their growth. The essential role of PR on the mammary gland became evident when [Lydon et al. \(1999\)](#) developed the PR knockout mouse and showed absence of mammary gland development or mammary tumor development in the absence of PR. The present therapy for endocrine-related breast cancer involves targeting ER or its ligands ([Jordan & Brodie 2007](#)). Since an important role for ER is the induction of PR expression, it seems reasonable to expect that anti-progesterin therapy may be a beneficial treatment approach for endocrine-responsive breast cancer. It seems possible that the use of therapies aimed to block the PR, either together or alternated with anti-estrogens or aromatase inhibitors may delay the onset of hormone resistance. An imbalance of PR isoform expression has been reported in breast cancers ([Mote et al. 2002](#)) and tumors with higher PR-A/PR-B ratios are those which have been shown to exhibit a poorer response to tamoxifen treatment ([Hopp et al. 2004](#)). Interestingly, in the MPA-induced tumor model, tumors with these features would respond to

antiprogesterins, and it may also be possible to identify similar subpopulations of human cancers.

The MPA breast cancer model has limitations and advantages. As a limitation common to other transplant models, neoplastic cells are injected into otherwise normal organisms in which the predisposing risk factors are absent. Also, the fact that most of the genetic engineered mice had been developed in backgrounds other than BALB/c, limits the possible use of these tumors in genetically modified backgrounds.

Probably, the most important advantages are that the model is established in immunocompetent animals and that the ductal mammary carcinomas originated express ER and PR, are hormone-responsive, and metastasize to lymph nodes and lungs. Even though we have not yet used this model for intracranial or intrafemoral tumor cells injections, this possibility remains to be exploited.

Established and highly characterized cell lines of human origin such as MCF-7, T47D, MDA-231, and others have become a standard tool in the study of different aspects of breast cancer. Each represent, however, what is found in a single individual, and recreating specific aspects such as hormone responsiveness/resistance usually require complex gene manipulations. We believe that the MPA model, with the ample array of carcinomas of different biological behavior, may better represent through their natural genetic drift, the interindividual variations and the heterogeneity found among tumors. It is also worth pointing that, regardless of this natural genetic drift and the acquisition by some of the tumors of specific mutations, hormone responsiveness remains closely similar in the whole family of parental tumors; most of them are still strongly hormone responsive. This allows the testing of different approaches to understand the acquisition of HI and hormone resistance, one of the most challenging areas of breast cancer research.

The possibility to work with primary cultures in 2D and 3D, separating CAF from epithelial cells or co-culturing both populations has allowed us to investigate the role of tumor stroma on the acquisition of HI. In addition, undergoing studies profiling stroma from different HD and HI tumors growing *in vivo* will provide further information to understand the role of stroma in HI and resistance. It has recently been hypothesized that progesterins may induce the selection of stem cell in patients with breast cancer (Horwitz & Sartorius 2008), which may open up the possibility of investigating this hypothesis in the MPA-induced breast cancer model, and to investigate, whether we have targeted the stem cell population in tumors that have experienced complete clinical regression.

Standard concepts on general carcinogenesis fail to fit the natural history of hormone-induced mammary carcinomas, as effective proof of a specific mutagenic initiation hit is still lacking. Moreover, present knowledge points to the fact that the progression of these cancers seems to be driven by epigenetic events and signaling pathway cross-talks, rather than by specific genetic changes. A revisit to the general cancer paradigm is in this case, long overdue. The MPA-induced mammary carcinomas may be the ideal environment in which to test these highly relevant questions, as it represents a hormone-induced, hormone-driven cancer that progresses strongly associated with the EGF signaling axis in the preneoplastic stages, becomes addicted to the PR pathway, and closely interacts with other relevant paths such as that of the FGFR.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This project has been supported by grants from the Sales Foundation, and also by grants from SECYT, CONICET, and the Roemmers Foundation. Dr Molinolo is supported by the Intramural Research Program of the Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA.

Acknowledgements

We wish to thank Dr Christiane Dosne Pasqualini for being the mentor of this model and Dr Silvio Gutkind (NIDCR, NIH) for critically reading the manuscript and for helpful suggestions. We also thank Miss Julieta Bolado and Dr María Alicia Gorostiaga for excellent technical support, Gador Laboratories (Buenos Aires) for providing MPA and to Raffo Laboratories (Buenos Aires) for the doxorubicin liposomes. We also thank Bayer Schering Pharma AG, Berlin for the ZK 230211, Pfizer Global Pharmaceuticals for the PD 173074 and to Zeneca Pharmaceuticals England for kindly providing fulvestrant.

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