REVIEW

Biological and clinical impact of imbalanced progesterone receptor isoform ratios in breast cancer

Caroline A Lamb[1], Victoria T Fabris[1], Britta M Jacobsen[2], Alfredo Molinolo[3] and Claudia Lanari[1]

[1]Instituto de Biología y Medicina Experimental (IBYME), CONICET, Buenos Aires, Argentina
[2]University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA
[3]Moore's Cancer Center, UCSD, La Jolla, California, USA

Correspondence should be addressed to C Lanari: lanari.claudia@gmail.com

Abstract

There is a consensus that progestins and thus their cognate receptor molecules, the progesterone receptors (PRs), are essential in the development of the adult mammary gland and regulators of proliferation and lactation. However, a role for natural progestins in breast carcinogenesis remains poorly understood. A hint to that possible role came from studies in which the synthetic progestin medroxyprogesterone acetate was associated with an increased breast cancer risk in women under hormone replacement therapy. However, progestins have also been used for breast cancer treatment and to inhibit the growth of several experimental breast cancer models. More recently, PRs have been shown to be regulators of estrogen receptor signaling. With all this information, the question is how can we target PR, and if so, which patients may benefit from such an approach? PRs are not single unique molecules. Two main PR isoforms have been characterized, PRA and PRB, which exert different functions and the relative abundance of one isoform with respect to the other determines the response of PR agonists and antagonists. Immunohistochemistry with standard antibodies against PR do not discriminate between isoforms. In this review, we summarize the current knowledge on the expression of both PR isoforms in mammary glands, in experimental models of breast cancer and in breast cancer patients, to better understand how the PRA/PRB ratio can be exploited therapeutically to design personalized therapeutic strategies.

Key Words

- progesterone receptor isoforms
- progestins
- antiprogestins
- breast cancer
- patient-derived xenografts
- PR isoform ratio
- prognostic markers
- *in vivo* breast cancer models

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Introduction

Nuclear estrogen receptors alpha (ERα) represent almost the first molecular targets used for breast cancer treatment, and two-thirds of these patients are treated with an endocrine therapy. Nuclear progesterone receptors (PRs) on the other hand, have been evaluated as surrogate markers of ERα integrity and functionality. There is nowadays emerging interest in understanding the interplay between the different members of the nuclear hormone receptor family and their role in tumor growth.

PRs are members of the nuclear steroid family of receptors, together with ER, androgen (AR), glucocorticoid (GR)/mineralocorticoid (MR) receptors and other less related molecules such as thyroid receptors, retinoic acid and orphan receptors (reviewed in Grimm *et al.* 2016).

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They are ligand-activated transcription factors that can also act through ligand-independent mechanisms. Receptors other than the classical nuclear steroid receptor family are also involved in generating progesterone (P4)-mediated effects (reviewed in Mueck *et al.* 2014).

PR isoforms

The human PR gene consists of eight coding exons separated by seven non-coding introns. There are mainly two mRNA transcripts controlled by two different promoters, each one encoding a different protein. The mRNA regulated by the distal promoter encodes the full-length PR named PRB (116 kDa; 933 amino acids (aa)); the one regulated by the proximal promoter encodes the truncated version, named PRA (94 kDa), which lacks the first 164 aa starting its translation in the methionine 165 (reviewed in Abdel-Hafiz & Horwitz 2014). These two isoforms were originally described in the chick oviduct. Other mRNA PR variants with different deleted exons were detected in breast cancer tissues and cell lines (Cork *et al.* 2008). However, how these mRNAs influence PR signaling and if they are translated into functional proteins remains to be established. A third predicted truncated PR isoform, named PRC (60 kDa), results from an alternative translation site starting at methionine 595. This form should retain the ability to bind the ligand, but not DNA. It was first described in T47D cells (Wei *et al.* 1990), and it has been suggested that it may play a role in labor (Condon *et al.* 2006) where high PRC levels are associated with an increase in the PRA to PRB ratio (Renthal *et al.* 2015). A 78 kDa protein was also detected in breast cancer samples using PR antibodies (Graham *et al.* 1995). Since it is not reactive with an antibody that recognizes only PRB, it has been originally proposed as a PRA variant (Yeates *et al.* 1998).

In the next part of this review, we will focus on the two main PR isoforms, PRB and PRA, and the impact that the different PR isoform ratios might have on breast cancer progression.

PRA and PRB isoforms: localization, genomic and non-genomic effects

PRB isoforms have an amino terminal region named A/B, with a high degree of variability among the different nuclear receptors. Two transactivation domains: AF1 (aa 456–546) and AF3 (aa 1–164) located in this region participate in coactivator recruitment (Dobson *et al.* 1989). In addition, an inhibitory domain is involved in corepressor recruitment (Hovland *et al.* 1998). The DNA-binding domain consists of 78–80 aa and is composed of two type II zinc finger structures involved in the binding to specific cis-acting DNA sequences, the PR elements (PRE). The ligand-binding domain is located at the carboxy terminal end of the PR. It contains a third transactivation domain (AF-2) required for hormone-dependent coactivator recruitment, dimerization and interaction with chaperone proteins in the inactive state (reviewed in Hilton *et al.* 2015).

PRA differs from PRB in the lack of the AF3 transactivation site, located in the B-Upstream Segment (BUS) of PRB (Sartorius *et al.* 1994*b*). Similar affinities of PRA and PRB for the natural ligands and medroxyprogesterone acetate (MPA) have been reported (Ghatge *et al.* 2005); however, this is not valid for the progestins levonorgestrel or gestodene (Schoonen *et al.* 1998). After ligand activation, PR isoforms dimerize forming homo- or heterodimers. There is still no consensus on whether PR dimerization is necessary for binding to palindromic DNA sequences. A different model proposes that the activation of single monomers, which bind to PRE half sites (Jacobsen *et al.* 2009), might be thermodynamically favored (Heneghan *et al.* 2005). Many of the endogenous PR-binding sites detected by chromatin immunoprecipitation sequencing contain these PRE half sites (Ballare *et al.* 2013). More recently, it has been shown that in cells transfected with PRB, ligand activation promotes tetramerization of PR in a DNA-independent manner (Presman *et al.* 2016).

Another level of complexity is the fact that P4 may also elicit rapid non-genomic effects activating cytoplasmic signaling pathways (reviewed in Boonyaratanakornkit *et al.* 2018). These effects are mediated by PRB, as this isoform shuttles between nuclear and cytoplasmic compartments whereas, PRA is mainly localized in the nucleus (Guiochon-Mantel *et al.* 1994, Boonyaratanakornkit *et al.* 2008). In pathological contexts, the abnormal presence of cytosolic PRA may interfere with normal signaling.

PRA may also act as a ligand-dependent trans-dominant repressor of other steroid receptors including PRB, ERα, AR, MR and GR (reviewed in Chabbert-Buffet *et al.* 2005), but this effect may not be so relevant under physiological conditions (Scarpin *et al.* 2009). In most cases, PRB functions as an activator of progesterone-responsive genes in conditions in which PRAs are transcriptionally inactive (reviewed in Jacobsen & Horwitz 2012). Notwithstanding, genes regulated exclusively by PRA have been reported (Richer *et al.* 2002, Jacobsen *et al.* 2005). PRB competes with ERα for the same coactivators (McDonnell & Goldman 1994, Wen *et al.* 1994).

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This coactivator squelching represents another mechanism through which progestins inhibit estrogen action. More recently, a rewiring of ERα and PR after progestin treatment was proposed to explain the inhibitory effects of progestins on breast cancer cell proliferation (Mohammed *et al.* 2015).

After ligand binding, PR aggregates in nuclear foci where the transcriptional activity takes place (Arnett-Mansfield *et al.* 2007). The activated PR exerts their genomic effects by binding either to PRE directly at the promoter of target genes or can be tethered to other transcription factors, thus regulating the expression of genes that lack PRE sites. PR isoform-mediated transcription has been reviewed in depth elsewhere (Jacobsen & Horwitz 2012). Post-translational modifications can modulate PR transcriptional activity. These include phosphorylations, acetylations, ubiquitinations, glycosylations and SUMOylations in serine and lysine residues located in the amino terminal end. The functional effects of these modifications are related to several factors such as modulation of the transcriptional activation or repression, DNA binding, cofactor recruitment, receptor turnover and changes in hormone responsiveness (reviewed in Qiu & Lange 2003, Abdel-Hafiz & Horwitz 2014, Diep *et al.* 2015). Several studies have suggested that, upon ligand binding, PRA is rapidly SUMOylated, while PRB becomes deSUMOylated. SUMOylation is a stabilizing modification and thus PRA results in a more stable isoform relative to PRB (Daniel *et al.* 2007). This biochemical difference in isoform SUMOylation, together with the increased turnover of PRB due to mitogen-activated protein kinase-dependent phosphorylation of serine 294 (Shen *et al.* 2001, Faivre & Lange 2007) may explain the PRA predominance observed in many human data sets reviewed herein.

Methods to determine PR isoform expression

PR isoform expression has been determined using PCR, western blots (WB) or immunohistochemistry (IHC)/immunofluorescence (IF) techniques. A critical issue in PR isoform mRNA detection is the correct primer design. Errors were detected in several studies by Aupperlee *et al.* (2005*a*). The levels of PRA are calculated by subtracting PRB from total PR adding a further degree of complexity.

Currently, WB is the most reliable method to evaluate PR isoforms since the ratio can be calculated in the same assay using one antibody. Notwithstanding, there are several limitations: (a) PRB splice variants with similar molecular weight (MW) to PRA might interfere with the PRA band; (b) antibodies should have equal affinities for both denatured PR proteins and (c) WB is not in the routine practice in the clinic.

The detection of PR isoforms by IHC or IF is still controversial. A list of antibodies that did not recognize PRB by IHC in gelatin-embedded cells uniquely expressing PRB has been reported (Mote *et al.* 2001). A PRA-specific antibody is offered by Leica, a Novocastra antibody known as clone 16 or 312. The FDA approved this antibody to detect total PR in human tissue; however, the antibody is commercialized as a specific PRA antibody. Following these assumptions, many investigators have published results describing and quantifying PR isoform expression in different tissues using IHC (Kreizman-Shefer *et al.* 2014, Bartosch *et al.* 2015, Bonneterre *et al.* 2016). In xenografts, using cell lines modified to express either PRA or PRB, this antibody proved to be excellent to recognize both PR isoforms by IHC (Fabris *et al.* 2017).

PR isoforms in the mammary gland

PRA and PRB expression ratio may change in the different reproductive tissues. Even in the same organ, the PR ratio may differ according to the developmental stage or hormonal status (reviewed in Patel *et al.* 2015, Hilton *et al.* 2018).

Both PR isoforms are expressed in the mammary gland of most of the species studied. Equimolar ratios have been reported in human breast (Mote *et al.* 2002), higher levels of PRA than PRB (3:1) in mouse mammary glands (Schneider *et al.* 1991), and a single PRB isoform in the rabbit. Table 1 depicts in detail the PRA/PRB changes during the different stages of mammary gland development in different species.

PR isoforms in genetically modified mice

PRB is required for mammary morphogenesis during pregnancy (reviewed in Conneely *et al.* 2003). PR-knockout (KO) mice show decreased branching and lobuloalveolar differentiation. PRA-KO mice are infertile, but the mammary gland develops normally in response to P4, indicating that PRB is necessary and sufficient to induce mammary gland proliferation and differentiation during pregnancy. PRB-KO mice are fertile but the mammary gland is unable to differentiate (reviewed in Conneely *et al.* 2003). Conversely, transgenic mice overexpressing PRA show ductal hyperplasia (Shyamala *et al.* 2000) that was reversed by antiestrogen but not with antiprogestin treatment (Sampayo *et al.* 2013) and, mice overexpressing

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Table 1 Expression of PR isoforms in different stages of development of normal mammary gland.

Species	Receptor	Puberty	Virgin adulthood	Pregnancy	Lactation	Antibody	Assay	References
Human	PR total	NAI	Luminal cells: 10–20% (constant throughout the menstrual cycle); PRA > PRB in BRCA1/2+	NAI	NAI	AB52 (from Dr K Horwitz) PR-86 hPRa3, NM*	IHC	Mote *et al.* (2002), Kariagina *et al.* (2008), Taylor *et al.* (2009)
	PRA	NAI	Luminal epithelial cells: 32.2%, co-expressed with PRB (1:1)	Luminal epithelial cells 6.2%; co-expressed with PRB	NAI	hPRa1, hPRa5 and hPRa7, NM Clone 16, cat NCL-PGR-312, NC* sc-538 (C-19) and sc-539 (C-20), SC*	IHC	
	PRB	NAI	Luminal epithelial cells/isolated myoepithelial cells: 23.5%. Co-expressed with PRA (1:1)	Epithelial cells 14.2% – also in isolated myoepithelial cells	NAI	hPRa6, NM NCL-PGR-B, NC	IHC	
Mouse	PR total							Aupperlee *et al.* (2005*b*), Montero *et al.* (2007), Kariagina *et al.* (2008)
	PRA	3 weeks old: 55% epithelial cells 6 weeks old: 58% of epithelial cells internal layer of end buds and ducts	Ductal cells: 50% in 12 weeks old/28% in 17–20 weeks old (constant throughout the estrous cycle) PRA:PRB 3:1	11% ductal; 6% alveolar cells. BALB/c > C57BL/6	ND	hPRa7, NM C-19, SC C-20, SC	IHC, IF	
	PRB	ND	ND	Alveolar cells (mostly): 48%	ND	hPRa6, NM	IHC, IF, WB	
Rat	PR total							Kariagina *et al.* (2007)
	PRA	30–40% epithelial cells in end buds, ducts and lobules (constant throughout the estrous cycle)	Ducts and lobules: 25% epithelial cells (constant throughout the estrous cycle). Co-expressed with PRB	Lobules: 5–10%; ducts: 20%	ND	A0098 (Dako) hPRa7 (NM)	IF	
	PRB	50–60% cells of cap cell layer of end buds, ductal and lobular cells. Expressed in epithelial (nuclei) and myoepithelial cells (nuclei and cytoplasm). Constant throughout the estrous cycle). Co-expressed with PRA (60%)	Epithelial and myoepithelial cells of ducts and lobules: 40–60%. Constant throughout the estrous cycle. Co-expressed with PRA (50%)	Ducts and lobules: 50–60%	Isolated myoepithelial cells (nuclear and cytoplasmic staining)	B15 raised against a synthetic Peptide N-t, AB*	IF	

Monkey	PR total	NAI	Lobular cells: 4–17%; increases throughout the menstrual cycle; maximum in luteal phase. Ductal epithelial cells: 12–15%; constant throughout the menstrual cycle	Increased percentage of positive cells in ducts and lobules	ND	NCL-PGR-312, NC	IHC	Cheng *et al.* (2005)
	PRA	NAI	Ductal and lobular epithelial cells: 12–15%; constant throughout the menstrual cycle	Low expression between 2 and 7% cells and 1% in late pregnancy	ND	NCL-PGR-312, NC	IHC	
	PRB	NAI	Ductal epithelial cells: 12–15%; constant throughout the menstrual cycle; co-localization with proliferation marker (21% of cells)	Low expression between 2 and 7% cells and 1% in late pregnancy	ND	Clone SAN27, NC	IHC	
Dog	PR total	NAI	Intermediate and basal epithelial ductal cells. Co-expressed with proliferation markers	NAI	NAI	hPRa4, 5, 7, NM C-20, SC PR10A9, IM	IHC, WB	Lantinga-van Leeuwen *et al.* (2000), Gracanin *et al.* (2012)
	PRA	NAI	High expression in metaestrus, anoestrus, with progestin treatment	NAI	NAI		WB	
	PRB	NAI	Low expression; only in anoestrus; with progestin treatment	NAI	NAI		WB	

NAI, No Available Information; ND, not detectable; *AB, Affinity Bioreagents, Golden, CO; NC, Novocastra; IT, Immunotech; NM, Neomarkers; SC, Santa Cruz Biotech; PR, progesterone receptor; PRA, PR isoform A; PRB, PR isoform B; WB, Western blot; IHC, immunohistochemistry; IF, immunofluorescence.

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PRB did not show ductal elongation and no ends buds were developed (Shyamala *et al.* 2000).

Transgenic *C/EBPB* mice showed an altered pattern of PR isoform expression (Grimm & Rosen 2003), suggesting that C/EBPB plays a role in the regulation of PR. PR, but not ERα, are also overexpressed in the mammary epithelial cells of *Brca1/p53*-deficient mice because of a defect in PR degradation (Poole *et al.* 2006), mirroring the data of human mammary glands from *BRCA*+ patients showing increased levels of PRA/PRB (King *et al.* 2004). Furthermore, treatment of *Brca1/p53*-deficient mice with the PR antagonist, mifepristone (MFP; RU-486) prevented mammary tumorigenesis (Poole *et al.* 2006).

PR isoforms in human breast cancer cell lines

PR isoform expression and PR isoform ratio differentially affect breast cancer progression. Although controversial, *in vitro* (Lin *et al.* 1999, Khan *et al.* 2012, Wargon *et al.* 2015, Diep *et al.* 2016) and *in vivo* (Sartorius *et al.* 2003, Wargon *et al.* 2015) studies with breast cancer cell lines support this notion.

The *in vitro* evidence comes from studies using: (a) T47D cells with constitutive expression of both PR isoforms or modified to alter the PR isoform ratio (McGowan & Clarke 1999, Jacobsen *et al.* 2002, McFall *et al.* 2015, Wargon *et al.* 2015) or engineered to overexpress only PRA (T47D-YA) or PRB (T47D-YB) (Sartorius *et al.* 1994*a*); (b) MCF-7 cells in which endogenous PR is expressed after estradiol treatment or modified to express exogenous PR (Boonyaratanakornkit *et al.* 2001, McGowan *et al.* 2007); (c) ZR-75-1 cells in which PRB is the prevalent isoform (Diep *et al.* 2016); (d) IBH-6 cells that express low ERα and PR levels (Vazquez *et al.* 2004) and have been modified to overexpress PRA or PRB (Wargon *et al.* 2015); (e) MDA-MB-231, which are claudin low triple-negative cells, modified to express both PR isoforms or only PRB (Lin *et al.* 1999, 2000) or to conditionally express PRA, PRB or both isoforms (Khan *et al.* 2012, Bellance *et al.* 2013) and (f) BT-474 cells modified to express higher PRB levels as compared to the PRA isoform (Wu *et al.* 2004).

Experimental evidence points to PRB as the more proliferative isoform (Faivre *et al.* 2008) (reviewed in Lange *et al.* 2008). Progestins induce cell cycle progression in T47D-YB cells stably expressing PRB (Skildum *et al.* 2005). However, a biphasic effect of progestins has been reported in T47D cells showing a late inhibitory effect preceded by an increase in cell cycle progression (Musgrove *et al.* 1991). Conversely, in T47D cells with inducible PRA, there was no significant change in cell proliferation but displayed reduced adherence to plastic, suggesting that increased PRA expression, may play a role in loss of adhesion observed in cancers (McGowan & Clarke 1999, Graham *et al.* 2005) and an increased ability to invade stromal tissue (McGowan *et al.* 2004).

The experimental data regarding the role of PR isoforms in tumor invasiveness and/or aggressiveness *in vitro* is still controversial. In some experimental models, PRA+ cells exhibit a more invasive and aggressive behavior (McGowan *et al.* 2003, Jacobsen *et al.* 2005), whereas in others, induction of PRB leads to an increase in cell migration (Ibrahim *et al.* 2008, Bellance *et al.* 2013).

The genomics of P4-induced effects on gene transcription mediated by PR isoforms has been studied in the T47D-YA/-YB model (Richer *et al.* 2002, Tung *et al.* 2006), in the MDA-MB-231 cells transfected with total PR or PRB (Leo & Lin 2008) or in the inducible systems (Graham *et al.* 2005, Jacobsen *et al.* 2005, Khan *et al.* 2012). In this review, we will focus on data obtained *in vivo*.

PR isoforms in experimental tumor growth

Mouse mammary carcinomas

There are few mouse mammary carcinoma models that express ERα and PR. MMTV-induced tumors were studied at a time that PR was detected by ligand-binding techniques and, to the best of our knowledge, no information has been reported regarding the prevailing PR isoforms in these models.

Our group developed MPA-induced mammary carcinomas, which expressed high levels of ERα and PR (reviewed in Lanari *et al.* 2009). With time, several different tumor variants arose which were classified according to their response to antiprogestin treatment. PR isoform expression revealed that responsive carcinomas had high PRA/PRB ratios while resistant carcinomas displayed the opposite ratio (Wargon *et al.* 2015). Two types of resistant variants were characterized: constitutive and acquired. The former carried a specific PRA promoter methylation and, upon demethylation, PRA expression and antiprogestin responsiveness were restored (Wargon *et al.* 2011). In acquired resistant variants, continuous antiprogestin treatment induced a decrease in PRA expression and estrogen or tamoxifen treatment restored antiprogestin responsiveness by increasing PRA expression, thus changing the PRA/PRB ratio (Wargon *et al.* 2009).

A large number of genetically engineered mice have also been developed in an attempt to model endocrine-responsive human breast cancer; however, few ER+

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luminal mammary carcinomas have been generated (reviewed in Dabydeen & Furth 2014). PR isoforms were only briefly mentioned. In the *Wnt1* transgenic model, variable levels of PRB and PRA were observed among the different tumors (Zhang *et al.* 2005); whereas in the two cases shown of the *Stat1* KO model, high levels of both PRA and PRB were observed in WB, although they were not quantified (Chan *et al.* 2012).

Rat mammary carcinomas

To the best of our knowledge, there are no available data on PR isoform expression in rat mammary carcinomas.

Feline mammary carcinomas

The percentage of total PR+ cells increases in feline invasive mammary carcinomas as compared to normal mammary gland (67% vs 15%, respectively) (Millanta *et al.* 2005) and similar levels of PRA and PRB were observed by WB in mammary carcinomas (Gracanin *et al.* 2012).

Canine mammary carcinomas

In PR+ canine mammary carcinomas, higher levels of PRA than those of PRB were observed (Gracanin *et al.* 2012, Guil-Luna *et al.* 2014) and the antiprogestin aglepristone inhibited tumor growth (Guil-Luna *et al.* 2011). More recently, tumor samples excised before and after aglepristone treatment were analyzed for PR mRNA and Ki67 antigen labeling (Guil-Luna *et al.* 2017). In aglepristone-treated PRA+ tumors, both total PR and PRA mRNA expression levels decreased as well as the proliferation index, suggesting that PRA mediates the inhibitory effect.

Cell line-derived xenograft models

The PRA/PRB ratios in different breast cancer cell line-derived xenograft models are depicted in Table 2. T47D xenografts are strictly 17-β-estradiol (E2)-dependent for their *in vivo* growth. Bagatell *et al.* reported double bands for PRA and PRB using the PR MC243 antibody in WB (Bagatell *et al.* 2001), whereas Sartorius *et al.* showed increased PRB expression as compared with that of PRA in the T47D xenografts growing in nude female mice (Sartorius *et al.* 2003). We found similar levels of both PR isoforms, but occasionally there was an increase in PRB. T47D and T47D-YA tumors grow slower than the T47D-YB when inoculated into nude (Sartorius *et al.* 2003) or NSG mice (Wargon *et al.* 2015). T47D-YA xenografts are tamoxifen and MFP sensitive while T47D-YB are resistant to both agents (Sartorius *et al.* 2003, Wargon *et al.* 2015). MPA induced only a slight increase in tumor growth of T47D xenografts; however, it did increase the expression of stem cell markers such as CK5 and BCL6 (Goodman *et al.* 2016). Others reported that P4 slightly decreased estrogen-induced tumor growth in MCF-7 and in T47D xenografts (el Etreby & Liang 1998, Mohammed *et al.* 2015) although in the latter, the decrease in growth rate was only significant when P4 was administered together with tamoxifen.

More recently, Singhal *et al.* reported that in T47D xenografts, the antiprogestin telapristone (TLP) induced transient inhibition of tumor growth, while the combined administration together with tamoxifen induced an almost complete tumor regression (Singhal *et al.* 2016). A similar effect was obtained with two other antiprogestins: CDB 4453 and EC313 (Singhal *et al.* 2018). These results are in line with the inhibitory effect previously observed in T47D xenografts treated with MFP (Wargon *et al.* 2015).

P4 (pellet; 25 mg) inhibited the growth of MDA-MB-231 cells transfected with PR (ABC28 clone) inoculated into ovariectomized NOD/SCID mice. Overall the tumors were too small, and no metastases were observed in any of the groups (Lin *et al.* 2001). Recently, we found that tumor growth and metastases were lower in MDA-MB-231 cells stably transfected with PRB inoculated into NSG mice as compared to the control counterparts. However, while MPA inhibited lung metastasis, MFP induced an increase in the number and size of the metastatic foci (Lanari *et al.* 2016).

IBH-4, IBH-6 and IBH-7 are ERα+ PR+ human breast cancer cell lines (Vazquez *et al.* 2004) that originate tumors when transplanted into nude mice (Bruzzone *et al.* 2009). IBH-4 and IBH-6 were able to grow without hormone supply and IBH-7 was strictly estrogen dependent (Bruzzone *et al.* 2009). Tumors express ERα and PR and although not quantified, higher levels of PRB than PRA were observed in WB (Bruzzone *et al.* 2009). MPA did not alter tumor growth; however, in the three cases the curves corresponding to MPA-treated mice showed a trend to decrease the growth rate as compared with their matched controls, either in the presence or absence of E2. Tamoxifen significantly inhibited the growth of IBH-4 and IBH-6 tumors.

IBH-6 tumors were not inhibited by antiprogestins such as MFP or TLP. However, these antiprogestins inhibited the growth of cells manipulated to overexpress PRA, whereas MFP stimulated those overexpressing PRB (Wargon *et al.* 2015).

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Table 2 PR isoform imbalance in cell line xenograft breast cancer models.

Cell line/tumor	PRA/PRB ratio	Assay	Mice	Antibodies	Effect of hormones/ antagonists	References
T47D (ER+, PR+, AR+, GR+ (low), HER2−, P53 mutation)	PRA = PRB[1,2] PRB > PRA[3]	WB	NSG[1,6] SCID[2] *Nu/nu*[3,4,5,7]	MC243[#2]; AB-52* B-30*[3] H-190 (sc-7208, SC[€]) or Dako 1294[1]	MPA ↑ tumor growth[4] and stem cell markers[5] P4 ↓ slightly E2-induced tumor growth and improved TAM-induced inhibition[6] Antiprogestins (MFP[1], TLP[1,7]) ↓ tumor growth Antiprogestins (CDB4453 and EC313) ↓ tumor growth that is potentiated by TAM[8]	1: Wargon *et al.* (2015); 2: Bagatell *et al.* (2001); 3: Sartorius *et al.* (2003); 4: Liang *et al.* (2007); 5: Goodman *et al.* (2016); 6: Mohammed *et al.* (2015); 7: Singhal *et al.* (2016); 8: Singhal *et al.* (2018)
	T47D-YA Only PRA	WB	*nu/nu*[1] NSG[2]	AB-52; B-30[1]; H-190 (SC) or Dako 1294[2]	↓ growth rate as compared with -YB[1,2]; inhibited by TAM[1] and MFP[2]	1: Sartorius *et al.* (2003); 2: Wargon *et al.* (2015)
	T47D-YB only PRB	WB	*nu/nu*[1] NSG[2]	H-190 (SC) or Dako 1294	↑ growth rate as compared with wt and –YA[1,2]; TAM[1] and MFP[2] resistant	1: Sartorius *et al.* (2003); 2: Wargon *et al.* (2015)
MDA-MB-231 (ER−, PR−, AR−, HER2−, P53 mut)	Transfection of PRA/-B ABC28 clone	NAI	SCID	NAI	P4 ↓ tumor growth	Lin *et al.* (2001)
	PRB	WB	NSG	H-190 (SC)[1,2]; Clone 16 (NC[€])[2]; Clone 636 (NC)[2]	Fewer metastases than wt. MPA ↓ and MFP ↑ lung metastasis[1]	1: Lanari *et al.* (2016); 2: Fabris *et al.* (2017)
IBH-6 (ER+, PR+, HER2-)	PRB > PRA Wt	WB	*Nu/nu*[1,2]	C-19, sc-538 (SC) H-190 (SC)	Similar growth in E2- or MPA-treated or untreated mice. TAM ↓[1] and MFP ↑ tumor growth[2]	1: Bruzzone *et al.* (2009); 2: Wargon *et al.* (2015)
	PRA > PRB PRA transfection	WB	*Nu/nu*[1,2]	H-190 (SC)	MFP or TLP ↓ tumor growth Others not tested	Wargon *et al.* (2015)
	PRB > PRA PRB transfection	WB	*Nu/nu*[1,2]	H-190 (SC)	MFP ↑ tumor growth. TLP exerted no effect	Wargon *et al.* (2015)
IBH-4 (ER+, PR+, HER2-)	PRB > PRA	WB	*Nu/nu*	C-19 (SC)	Similar growth in E2- or MPA-treated or untreated mice. TAM ↓ tumor growth. MPA ↓ E2-induced metastases	Bruzzone *et al.* (2009)
IBH-7 (ER+, PR+, HER2-)	PRB > PRA	WB	*Nu/nu*	C-19 (SC)	E2-dependent. MPA ↓ E2-induced metastases	Bruzzone *et al.* (2009)
BT-474 (ER+, PR+, HER2+, p53 mut)	NAI				P4 ↑ tumor growth, MPA ↑ metastasis	Liang *et al.* (2010)
MCF-7 (ER+ PR inducible, RA+, GR+, HER2-P53 wt)	MCF-7 empty vector	WB (cell extracts[1])	NSG[1] *Nu/nu*[2]	1: NAI	P4 ↓ E2-induced tumor growth[1]. ONA, MFP or P4 ↓ as TAM tumor growth[2]	1: Mohammed *et al.* (2015); 2: el Etreby and Liang (1998)
	MCF-7 PRA	WB	NSG	NAI	Slower growth than wt	Mohammed *et al.* (2015)
	MCF-7PRB	WB	NSG	NAI	Slower growth than wt	Mohammed *et al.* (2015)

[#]Antibody provided by Dr D Toft, Mayo Clinic, Rochester, MN; *antibody developed by authors (Estes *et al.*, Biochemistry 26: 6250, 1987); [€]SC, Santa Cruz Biotech; NC, Novocastra; CS, Cell Signaling Technology; NAI, No Available Information; AR, androgen receptor; E2, 17-β-estradiol; ER, estrogen receptor; GR, glucocorticoid receptor; MFP, mifepristone; MPA, medroxyprogesterone acetate; ONA, onapristone; P4, progesterone; PR, progesterone receptor; PRA, PR isoform A; PRB, PR isoform B; TAM, tamoxifen; TLP, telapristone; WB, Western blot.

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Patient-derived xenografts

Only in few xenograft models, PR isoforms were evaluated by WB. In three out of four tumors of the WHIM xenografts, the PRB band seems stronger than the PRA band (in the publication the bands are mislabeled; the upper band is PRB and the lower band is PRA). More recently, the growth of two ERα+, PR+ patient-derived xenografts (PDX), UCD4 and UCD65 was described (Finlay-Schultz *et al.* 2017). P4 and MPA similarly inhibited E2-induced tumor growth. In both cases, PRB expression was apparently higher than PRA by WB (Finlay-Schultz *et al.* 2017). Esber *et al.* (2016) showed WB data from the HBCx-19, -21, -22 and -34 PDX (Cottu *et al.* 2014); both PR isoforms were expressed with levels of PRB that seemed to be higher than those of PRA. The authors report a slight inhibition of tumor growth after treatment with ulipristal acetate, a selective PRs modulator, P4 or the novel antiprogestin APR19 using the HBCx-34 PDX (Table 3).

In summary, increased levels of PRB than PRA were seen in most models in which progestins showed inhibitory effects on tumor growth or metastasis. Taking into consideration that the ability to generate a PDX is by itself a bad prognostic factor (DeRose *et al.* 2011, Byrne *et al.* 2017, Shafaee & Ellis 2017), it can be speculated that higher levels of PRB than PRA are associated with worse prognosis.

PR isoforms in breast cancer cohorts

ERα, PR, HER2 and Ki67 are routinely evaluated as prognostic and predictive markers in breast cancer patients. According to the ER status, patients are categorized in ERα+ PR+; ERα+ PR−; ERα− PR+ or ERα− PR−. Whereas there is no doubt regarding the clinical benefit of measuring ERα, the benefit of measuring PR expression is more controversial (Olivotto *et al.* 2004). However, as pointed out by several studies and discussed by many authors, PR expression has been proposed as a surrogate marker for ERα integrity and endocrine response since high total PR levels correlate with an improved tamoxifen response, longer disease-free and overall survival (Fuqua *et al.* 2005, MacGrogan *et al.* 2005). Moreover, the ERα+PR− group has a worse prognosis, and this phenotype has been associated with impaired ERα function or aberrant growth factor signaling that could contribute to tamoxifen resistance (Arpino *et al.* 2005). On the other hand, the existence of the ERα−PR+ group of breast cancer patients remains controversial. It has been proposed that in selected samples, either the lack of ERα staining or the detection of false-positive PR are related to technical issues, since in many cases which had been revisited a change in diagnosis has been registered (reviewed in Kunc *et al.* 2018). However, others sustain that ERα−PR+ tumors represent a different subgroup with distinct molecular features and clinical course (Shen *et al.* 2015).

In our hands, from 352 samples with ERα scoring, 258 (82.10%) were ERα+ PR+, 59 (16.76%) were ERα− PR−; 31 (8.80%) were classified as ERα+ PR−, and only three samples were reported as ERα− PR+ (0.09%). In all three cases, these samples displayed a PR staining ≤40% and PR isoforms were not detected by WB (unpublished data).

Only few studies have addressed the expression of the different PR isoforms in breast cancer (Table 4). The first study evaluating PR isoforms in breast cancers was reported in 1995 (Graham *et al.* 1995). They used 202 cytosols from PR+ primary tumors and analyzed the pattern of PR bands in WB. Several bands were observed; one around 115/120 kDa equivalent to the PRB band of T47D cells used as controls, another ahead from the 81 kDa band observed in the controls considered as PRA and one of 78 kDa. Extra faint bands with lower MW were occasionally observed. The median PRA/B ratio observed was 1.26.

Bamberger *et al.* studied a total of 53 mammary carcinomas, 21 PR− and 32 PR+ (Bamberger *et al.* 2000) by WB. Band intensity was quantified as negative, weak, moderate, strong and very strong. In only two out of 32 cases, one was a recurrence, PRB showed to be increased, as compared with PRA. In this study, the authors conclude that PRB expression correlates with the absence of HER2. Overall, the authors conclude that higher PRB levels correlated with a more differentiated phenotype. The drawback is the small cohort and the fact that the authors included in the data analysis all PR+ and PR− patients.

Ariga *et al.* evaluated the expression of PR by IHC (Ariga *et al.* 2001). The samples included 47 cases of invasive ductal carcinoma, 40 ductal carcinomas *in situ* (DCIS), 27 atypical ductal hyperplasias and 27 cases of proliferative disease without atypia. The IHC scores for PRA in atypical ductal hyperplasias were higher than those in high histological grade DCIS or invasive carcinomas, whereas the scores for PRB were lower in invasive carcinomas with high histological grades, than in proliferative disease without atypia. Both scores correlated inversely with histological grade in invasive carcinoma and DCIS. A positive correlation between ERα, PRA and PRB was observed in invasive carcinomas. Again, as in the previous study, the analysis was performed on PR+ and PR− samples. From the 47 cases of invasive carcinomas, 16 were PR− and 31 PR+.

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Table 3 PR isoform imbalance in breast cancer patient-derived xenograft models.

PDX	PRA/PRB ratio	Assay	Mice	Antibodies	Effect of hormones/antagonists	References
PDX WHIM 16 skin met	PRB > PRA	WB	NSG	#8757, CS[€]	E2 ↓ tumor growth	Li *et al.* (2013)
PDX WHIM 18 skin met	PRA > PRB	WB	NSG	#8757, CS	E2 has no effect on growth, FUL resistant	Li *et al.* (2013)
PDX WHIM 20 skin met	PRB > PRA	WB	NSG	#8757, CS	E2 has no effect on growth	Li *et al.* (2013)
PDX WHIM 24 skin met	PRB > PRA	WB	NSG	#8757, CS	E2 ↑ tumor growth	Li *et al.* (2013)
PDX UCD UCD4	PRB > PRA	WB	NSG	H-190 (SC); DAKO, 1294	E2 ↑ tumor growth; MPA or P4 ↓ E2-induced tumor growth	Finlay-Schultz *et al.* (2017)
PDX UCD UCD65	PRB > PRA	WB	NSG	H-190 (SC); DAKO, 1294	E2 dependent; MPA or P4 similarly ↓ E2-induced tumor growth	Finlay-Schultz *et al.* (2017)
HBCx 19	PRB > PRA	WB	*Nu/nu*	C-19 (SC)	NAI	Esber *et al.* (2016)
HBCx 21	PRB > PRA	WB	*Nu/nu*	C-19 (SC)	NAI	Esber *et al.* (2016)
HBCx 22	PRB > PRA	WB	*Nu/nu*	C-19 (SC)	NAI	Esber *et al.* (2016)
HBCx 34	PRB > PRA	WB	*Nu/nu*	C-19 (SC)	E2 ↑ growth. P4, APR19, a novel antiprogestin and UPA ↓ tumor volume and/or tumor weight	Esber *et al.* (2016)

[€]SC, Santa Cruz Biotech; NC, Novocastra; CS, Cell Signaling Technology; NAI, No Available Information; E2, 17-β-estradiol; FUL, fulvestrant; MPA, medroxyprogesterone acetate; P4, progesterone; PR, progesterone receptor; PRA, PR isoform A; PRB, PR isoform B; UPA, ulipristal acetate; WB, Western blot.

The three previous studies did not analyze the role of PRA and PRB as predictive factors for endocrine therapies. However, in 2004, Hopp *et al*. presented data suggesting that high PRA levels identify a subgroup of women with a poorer response to tamoxifen (Hopp *et al*. 2004). They evaluated frozen primary breast tumor specimens from a cohort of 297 axillary lymph node-positive patients; 119 received no adjuvant therapy after surgery and 178 were treated only with tamoxifen. Eighty-nine per cent were ERα+ and 69% PR+ as determined by binding techniques. Total extracts were prepared for WB. PRA and PRB levels in tumors were normalized to PRB levels in the T47D-positive control lysate used in the same immunoblot. The primary outcome was disease-free survival. The authors reinforce the fact that it is not possible to distinguish between both isoforms by IHC and that stromal cells will not interfere with the balance between both of them. The median PRA/PRB ratio was 0.96. The PR isoforms showed a significant inverse correlation with tumor size, S phase and number of positive nodes. Only in the tamoxifen-treated group, PRA expression higher than PRB correlated with decreased disease-free survival, suggesting that the determination of PR isoforms could help to predict tamoxifen responsiveness.

Pathiraja *et al*. reported an association of *PRA* promoter methylation with worse outcome in ERα+ breast cancer patients (Pathiraja *et al*. 2011). They selected 500 patients who received tamoxifen treatment and 500 without endocrine treatment. Most of the tumors had a small size (48%<2 cm) and 66% were node negative. Of the 227 ERα+, PR− tumors, *PRA* was methylated in 25.9% of the tumors and *PRB* in 28.5% suggesting loss of PRA or PRB was not a result of DNA methylation in regulatory regions of the PR genes. In the tamoxifen-treated group, overall survival was worse for patients with methylated *PRA* compared with non-methylated *PRA*. This finding conceptually contradicts the previous study (Hopp *et al*. 2004), since those patients with methylated *PRA* are supposed to be those with higher levels of PRB than PRA, and thus, better disease-free survival, although PR isoforms were not evaluated in this study.

Lindet *et al*. evaluated in 299 breast cancer samples the relation between mRNA expression of total (PRA+PRB) and PRB with different mRNAs of the HER/ERBB family and showed that they inversely correlated with aggressiveness (Lindet *et al*. 2012). Knutson *et al*. evaluated pSer294 expression in ten breast carcinomas. Phospho-Ser294 PRB was detected in five out of seven PR+ samples, suggesting that these tumors might be those with an active PR pathway (Knutson *et al*. 2012).

Mote *et al*. evaluated by IF the expression of both PR isoforms (Mote *et al*. 2015). PR expression was determined in tissue microarrays (TMAs) derived from the TransATAC cohort of patients (n=710) treated either with tamoxifen or with the aromatase inhibitor anastrozole, with a 10-year median follow-up. For cohort 1, sections of archival formalin-fixed and paraffin-embedded (FFPE) tissue were stained sequentially for PRB and then PRA, using a dual

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Table 4 PR isoform ratio in breast cancer patients.

Study	Assay	Antibodies	Methods	Results	References
1	WB	Mix of hPRa7, hPRa0 (NM€)	202 cytosols from PR+ primary tumors	PRA and PRB bands plus an extra one of 78 kDa. PRA/PRB ratio between 0 and 2 in 61.4%, and between 0 and 4, in 75.2% of tumors. PRA is the predominant isoform	Graham *et al.* (1996)
2	WB	NCL-PGR (NC€)	53 mammary carcinomas (21 PR−; 32 PR+). Band intensity quantification: negative, weak, moderate, strong, and very strong	In 30/32 cases, PRA ≥ PRB. PRB correlated with better differentiation. The expression of both PR isoforms correlated with ERα expression	Bamberger *et al.* (2000)
3	IHC PCR (6 cases)	hPRa7 for PRA and hPRa2 for PRB (NM)	47 IDC* (31 PR+), 40 DCIS**, 27 ADH#, and 27 PDWA*** six cases were evaluated for (PRA + PRB) or PRB mRNA expression	IHC scores for PRB were lower in high-grade IDC than in the PDWA. Positive correlation between ERα, PRA and PRB in the IDC	Ariga *et al.* (2001)
4	Dual IF staining	hPRa6 for PRB and hPRa7 for PRA (own antibodies)	13 normal breast tissues (FFPE); archival FFPE blocks of 15 PDWA, 15 ADH; 15 DCIS, and 39 malignant carcinomas	IC: 51% equimolar, 10% PRB and 39% PRA predominance. Loss of control of relative PRA/PRB expression is an early event in breast cancer	Mote *et al.* (2002)
5	WB	#1294 (Dako)	297 frozen tumors from lymph node+ patients. 119: no adjuvant therapy; 178: TAM-treated. Isoforms normalized to PRB levels of the T47D control lysate. The primary outcome was disease-free survival	PRA/PRB ratio ranged 0–31 with 72% of the tumors showing a ratio between 0.5 and 2. The PR isoforms showed an inverse correlation with tumor size, S phase, and number of positive nodes. Only in the TAM-treated group PRA > PRB correlated with decreased free survival	Hopp *et al.* (2004)
6	PR methylation	NA	500 TAM-treated patients and 500 without treatment. All tumors were ERα+ and 77% PR+. Most of them had small sizes and low rates of metastasis	In 25.9% of PR− tumors, *PRA* was methylated. In the TAM-treated group, overall survival was worse for patients with methylated *PRA*. PRA is silenced in luminal breast cancer patients with worse prognosis	Pathiraja *et al.* (2011)
7	qPCR (PRA + PRB) and PRB	NA	299 samples. mRNA expression of total PR, PRB and mRNAs of the HER/ERBB family	PR isoforms inversely correlated with tumor aggressiveness. No differences were recorded between total PR or PRB isoform expression and HER/ERBB expression	Lindet *et al.* (2012)
8	Dual IF staining	PRB: hPRa6 (NM) or SAN27 (NC). PRA: hPRa7 (NM) and clone 16 (NC)	Tissue microarrays from the TransATAC cohort ($n = 710$) patients treated with TAM or anastrozole, (10-year median follow-up). Tumors were categorized into: PRA/PRB ratios between 0.8 and 1.2; lower than 0.8 and higher than 1.2	PRA = PRB (53%); PRA > PRB (29%); PRB > PRA (18%). A significant association between PRB predominance and previous or current use of MHT was observed. TAM-treated, but not anastrozole-treated patients had a shorter time to distant relapse if their tumors had high PRA/PRB ratios	Mote *et al.* (2015)
9	IHC	PRA: clone 16 (#312; NC). PRB: clone SAN27 H.1H (NC)	Archived samples from 789 women with IC were analyzed for PR isoforms. Tumors were considered as activated PR (APR)+ if they had an aggregated pattern of nuclear foci staining	From 79% of PR+ tumors, 25% were PRA+ and APR+, and 23% PRB+ and APR+. The APR+ score was associated with higher grade. The authors proposed this assay to select patients with aggregated PR expression as candidates for an antiprogestin treatment	Bonneterre *et al.* (2016)

(Continued)

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Table 4 Continued.

Study	Assay	Antibodies	Methods	Results	References
10	WB	#1294 (Dako) and H-190 (SC€)	Nuclear and cytosolic extracts from 222 frozen PR+ breast cancer samples were run in 8% gels for electrophoresis. Samples were categorized according to the PRA/PRB ratio Tumors were equimolar (ratio: 1.2–0.83), PRA high (PRA-H) if PRA/PRB ≥1.2 or PRB high (PRB-H) if PRA/PRB ≤0.83	PR+ tumors: 52.3% were PRA-H and 28.8% PRB-H. Molecular profiling and data mining associated PRA-H tumors with the luminal A and PRB-H with the luminal B tumors. PRB-H tumors were associated with lower total PR values, greater tumor size, higher histological grade, higher Ki-67 expression and HER-2 expression. 100% of PRA-H samples (n=19) were inhibited with 10 nM MFP in tissue cultures	Rojas *et al.* (2017)
11	WB, qPCR	8757 (Cell Signaling)	53 ER+/PR+ ductal carcinomas, from the Cooperative Human Tissue Network. T47D cells overexpressing PRA were used as controls	21 samples out of 53 showed PR isoform bands in WB. PRA is the prevailing isoform. A correlation between protein and mRNA is reported. PRA expression correlated negatively with miR92a3p and positively with miR-26b5p expression. A role for PRA inhibiting the inhibitory effect of ER on metastases is proposed	McFall *et al.* (2018)

#ADH, Atypical Ductal Hyperplasia; *IDC, Invasive Ductal Carcinomas; **DCIS, Ductal Carcinoma *In Situ*; ***PDWA, Proliferative Disease Without Atypia; €NC, Novocastra; NM, Neomarkers; SC, Santa Cruz Biotech.
IC, Invasive Cancer; NA, NOT Applicable; MHT, Menopausal Hormone Treatment; ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded tissue; IHC, immunohistochemistry; MFP, mifepristone; PR, progesterone receptor; PRA, PR isoform A; PRB, PR isoform B; APR, activated PR; TAM, tamoxifen; WB, Western blot.

immunofluorescence technique, selective for PRA and PRB, that reflects relative levels of the two isoforms (Mote *et al.* 1999). In FFPE samples, the antibodies used for these studies had been previously recommended by this group to recognize only PRA (Mote *et al.* 2001) or PRB (Clarke *et al.* 1987). The mean PRA/PRB ratio was determined and 138 tumors were categorized into samples with PRA/PRB ratios between 0.8 and 1.2 (equimolar; n=73); PRA/PRB ratios lower than 0.8 (PRB predominance; n=18) and ratios higher than 1.2 (PRA predominance; n=40) in accordance with previous findings (Mote *et al.* 2002). A significant association between PRB predominance and previous or current use of menopausal hormone treatment was observed. Of the 16% (15/93) of patients who had never used menopausal treatment, almost half (7/15, 47%) had tumors expressing a high proportion of PRB. In addition, tamoxifen, but not anastrozole-treated patients had a significantly shorter time to distant relapse if their tumors had high mean PRA/PRB ratios, suggesting that the PRA/PRB ratio may be a discriminating factor in predicting response to these endocrine agents. The major drawback in this study is that the NCL-PGR-312 antibody used to detect PRA recognizes both PR isoforms in human xenografts (Fabris *et al.* 2017).

Bonnaterre and coworkers reported a novel immunohistochemical analysis to distinguish tumors in which PR is activated (Bonneterre *et al.* 2016). Based on previous studies (Mote *et al.* 2001), they proposed two subnuclear morphological PR distribution patterns indicative of transcriptional activation status: an aggregated pattern, formed by activated ligand-bound PR disposed in nuclear foci and a diffuse pattern. The authors propose that activated PR (APR) foci/aggregates in breast cancer cells can be used as a biomarker for antiprogestin responsiveness, namely onapristone (ONA). In this study, tumors are categorized according to their 'nuclear PR pattern' and correlate it with antiestrogen treatment outcomes. PR isoforms were detected by IHC in archived 801 specimens with previously determined ERα and PR status from women with early invasive breast cancer. APR– (APRneg) tumors are those that are either PR–, or, if they express PR, they have a diffuse staining. From 79% of PR+ tumors, 25% were PRA+ and APR+ and 23% were PRB+ and APR+. The APRA+ was associated with higher histological grade. The authors propose this assay to select patients with aggregated PR expression as candidates for an antiprogestin treatment. As mentioned before, the specificity of the PRA antibody has not been corroborated (Fabris *et al.* 2017).

Our laboratory has recently published results from a study involving 222 PR+ breast cancer samples (Rojas *et al.* 2017), which were categorized according to the PRA/PRB ratio, as measured by WB. Tumors were

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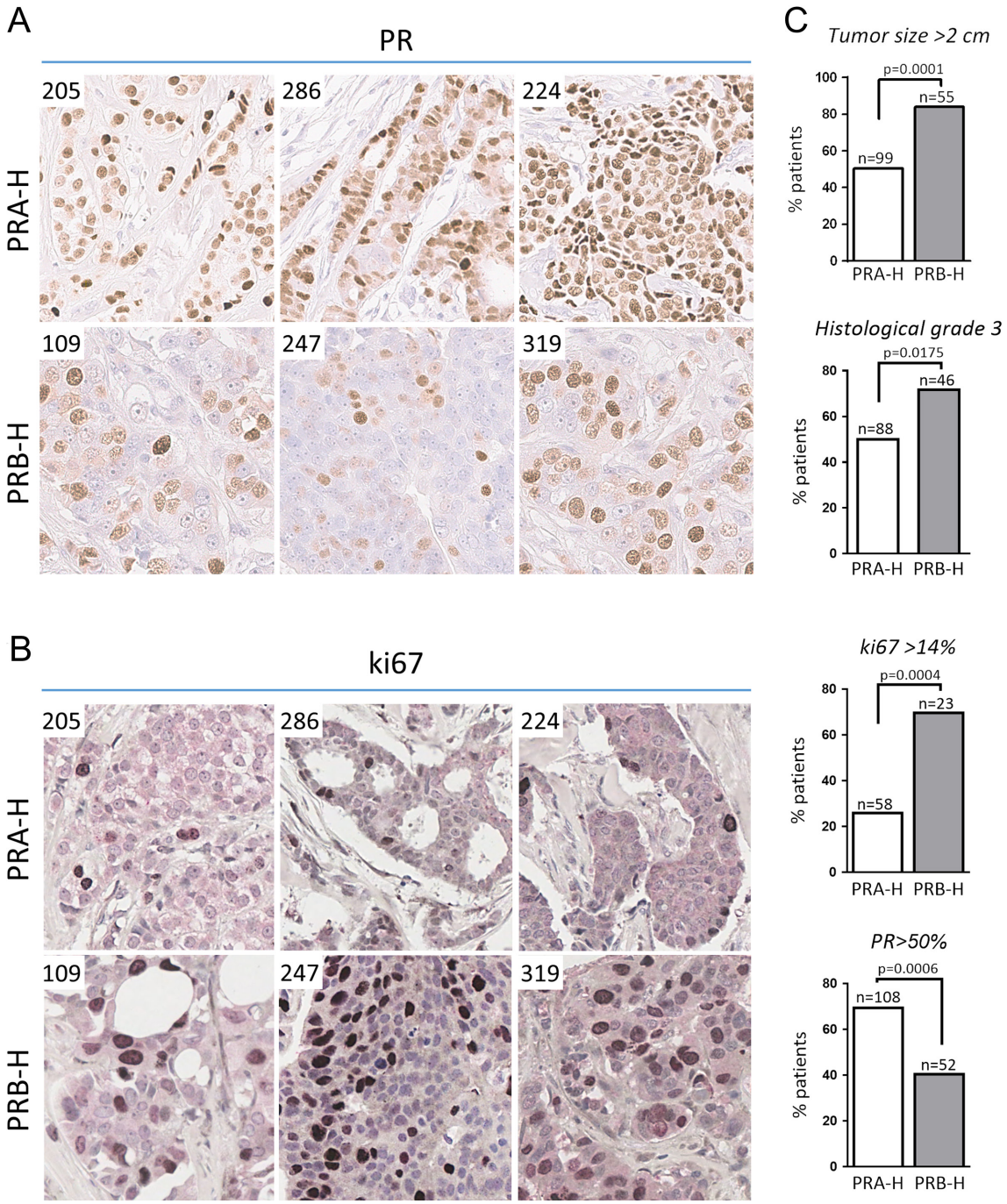

Figure 1
Quantification of clinicopathological parameters in PRA-H and PRB-H breast cancer samples. Immunohistochemical staining of PR (A) and ki67 (B) in three PRA-H and PRB-H breast cancer samples. (C) Quantification of the percentage of PRA-H or PRB-H patients with tumor sizes >2 cm, histological grade 3, Ki67 >14 or PR >50%. PR, progesterone receptor; PRA-H, breast cancer human sample with isoform A of PR/isoform B of PR ≥1.2; PRB-H, breast cancer human sample with isoform A of PR/isoform B of PR ≤0.83. The numbers on the corners in (A) and (B) correspond to the patient sample. Statistical analysis: Fisher exact test. Data obtained from Rojas *et al.* (2017). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0179.

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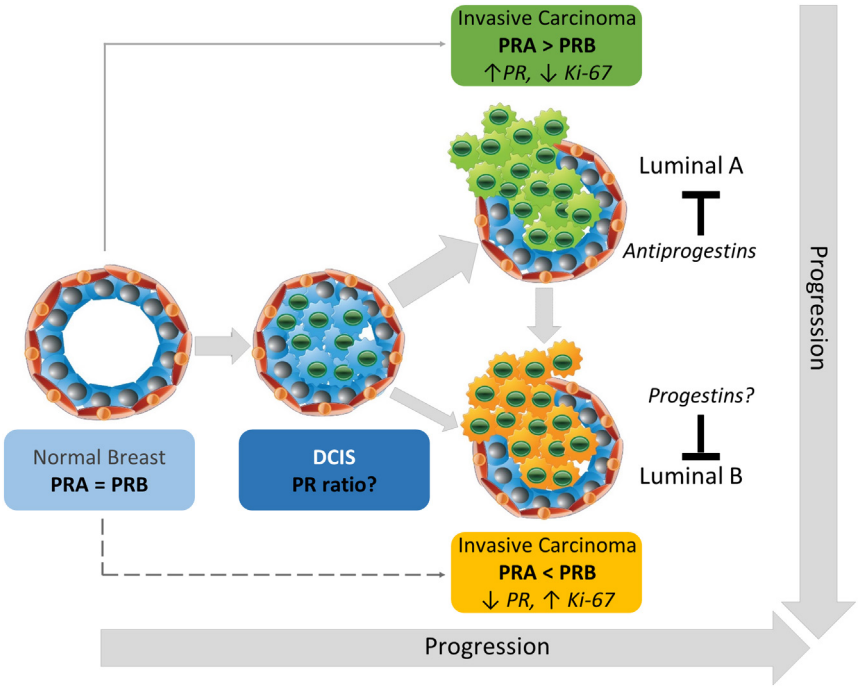

Figure 2
Schematic model of PR expression in breast cancer tumor progression. Few mammary epithelial cells express PR (equimolar PRA and PRB levels in the normal breast). Most invasive carcinomas express higher levels of PRA than PRB. PRA-H tumors recapitulate luminal A molecular subtypes (high total PR levels, low ki67 levels and respond to antiprogestin therapy). We hypothesize that invasive PRB-H tumors arise mainly during tumor progression. PRB-H tumors recapitulate luminal B molecular subtypes (low total PR levels, high ki67 levels, are resistant to antiprogestin therapy and may respond to progestin therapy). Invasive carcinomas may arise from DCIS (Cowell *et al.* 2013) or directly from mammary stem cells (Wang *et al.* 2013). DCIS, ductal carcinoma *in situ*; PR, progesterone receptor; PRA-H, higher levels of PRA than PRB; PRB-H, higher levels of PRB than PRA. A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0179.

considered equimolar if the ratio was within the range 1.2–0.83 or were classified as PRA high (PRA-H) if PRA/PRB ≥1.2 or PRB high (PRB-H) if PRB/PRA ≥1.2. In agreement with most of previous studies, PRA-H cases were predominant: 52.3% were PRA-H and 28.8% PRB-H, the rest being within the equimolar range. Molecular profiling and data mining of selected cases associated PRA-H tumors with the luminal A and PRB-H with the luminal B breast carcinomas. For this analysis, only ductal HER2– non-metastatic carcinomas were selected. This was corroborated by clinical parameters that associated the PRB-H with lower total PR values, greater tumor size, higher histological grade, Ki67 expression and HER-2 expression, supporting the data in molecular profiling (Fig. 1). Whereas in 100% of the PRA-H samples ($n=19$), a decrease in Ki67 staining was observed after incubation of tissue cultures with 10 nM MFP for 48 h, variable responses were observed in the 10 PRB-H, four equimolar or 3 PR– cases. This study is in line with that of Pathiraja *et al.* (2011), with observations of Knudson *et al.*, in modified T47D cells (Knutson *et al.* 2012) and with our preclinical studies suggesting that PRB-H tumors may be endocrine-resistant tumors (Lanari *et al.* 2012, Wargon *et al.* 2015).

Singhal *et al.* (2018) using the raw RNA-seq data from our study (Rojas *et al.* 2017) compared the list of differential genes expressed in T47D-YA and T47D-YB xenografts and those differentially regulated in PRA-H or PRB-H patients and concluded that a high PRA/PRB ratio might correlate with worse prognosis. Although the total number of samples included in both studies coincides, for the PAM50 analysis, we excluded the HER2+ cases, a lobular tumor and a metastatic tumor from a premenopausal patient, to compare a homogeneous cohort of patients. In Singhal's study, two of the HER2+ patients and the lobular patient were included, and a luminal A case of the PRA-H group was excluded. A larger cohort will be necessary to confirm these findings.

In a recent study, a positive correlation between PRA and miR26b5p expression and a negative correlation with miR92a3p was observed in 21 PRA+ breast cancer samples, with these two miRNAs being involved in the ER–PR crosstalk and in the invasive and metastatic features of luminal breast cancer (McFall *et al.* 2018). MiR92a expression is inversely correlated to tumor grade, positive lymph node status and recurrence-free survival in breast cancer (Nilsson *et al.* 2012).

In all these studies, the consensus indicates that PRA is the prevailing isoform in breast cancers; however, the role of the PRA/PRB ratio as a prognostic marker needs to be further validated in larger cohorts of breast cancer patients.

Clinical trials

Progestins (reviewed in Carroll *et al.* 2017) and antiprogestins (reviewed in Klijn *et al.* 2000, Lanari *et al.* 2012) have been used in the past to treat breast cancers with isolated positive responses. Among current clinical trials aimed to test the effects of antiprogestins

or progestins in breast cancer treatment, only the MIPRA trial (NCT02651844) has included the evaluation of the PR isoform ratio as an inclusion criterion to select patients for MFP treatment during 14 days between biopsy and surgery. Only those with PRA/PRB >1.5 are eligible. One study uses MFP for prevention in patients carrying *BRCA1* or -2 mutations with a high risk/incidence of breast and ovarian cancer (NCT01898312). As mentioned previously, *BRCA* carriers usually have levels of PRA higher than those of PRB (Fuqua & Cui 2004, King *et al.* 2004).

Other antiprogestins, such as ONA or TLP, are currently being tested. Regarding ONA (NCT02052128), although the study was expected to be completed by April 2016, the website has not been modified since June 24, 2015. With respect to TLP, the drug is given orally once daily for 2–10 weeks between biopsy and surgical resection (NCT01800422); the status is *active, not recruiting*. The NCT02314156 trial will evaluate transdermal or oral TLP in *BRCA* carriers undergoing mastectomy. The status is *active, not recruiting*. The NCT02408770 trial is also aimed to test the effect of antiprogestins in prevention and compares the effect of ulipristal acetate in breast density using MRI and the status is *unknown*.

On the other hand, a study using progestins has recently started. The NCT03306472 clinical trial is designed to evaluate the effect of letrozole alone or combined with megestrol acetate before surgery. The status is *recruiting*. Alternatively, the NCT00123669 trial consists of a unique dose of hydroxyprogesterone prior to surgery and the rationale is to test the effect of P4 in operable breast cancer on overall and disease-free survival at 5 years. The status is *active, not recruiting*.

Concluding remarks

PR ligands may stimulate or inhibit breast cancer growth, and the challenge is to determine which patient will respond to either treatment. Breast cancers with PRA/PRB ratios may respond to antiprogestin treatments, as supported by preclinical assays (Wargon *et al.* 2009, 2015), clinical evidence in dogs (Guil-Luna *et al.* 2014) and *ex vivo* data in human breast cancer tissue cultures (Rojas *et al.* 2017).

On the other hand, progestins may be inhibitory in tumors in which PRB levels are higher than those of PRA, as seen in preclinical assays (Wargon *et al.* 2015), in xenografted human breast cancer cell lines, and in PDX studies (Mohammed *et al.* 2015, Finlay-Schultz *et al.* 2017).

It has been suggested that most of the models in which progestins stimulate mammary carcinoma growth are biased because of the lack of ERα expression, as it is the case of the T47D-YA/YB models (Carroll *et al.* 2017) or because of the lack of estrogen supply. However, ERα levels in these models score higher than average ERα luminal breast carcinomas and experiments performed using syngeneic mice are performed under physiologic estrogen levels (Lanari *et al.* 2012).

Based on all the available information, malignant neoplastic transformation and the ability to invade neighboring tissues might be associated with an increased PRB activation resulting in PRB downregulation (Diep *et al.* 2015), inducing a switch from an equimolar stage to a preferential PRA-H stage in the human mammary gland. These tumors may be slow growing (low Ki67, luminal A) sensitive to ERα targeted therapy (and/or an antiprogestin treatment). As the tumor progresses and becomes insensitive to other treatments, there is an increase in cell proliferation, a decrease in total PR expression and mainly a decrease in PRA expression (which makes these tumors resistant to an antiprogestin therapy). We hypothesize that these high-grade tumors may now become sensitive to a progestin treatment (Fig. 2).

Future directions

More experimental work is necessary to better understand the role of PR isoforms in breast cancer growth and to exploit these receptors as therapeutic targets. Efforts should be geared to:

a. Design studies using homogeneous cohorts of patients to further define the PRA/PRB ratio as a prognostic factor.
b. Avoid the use of antibodies that supposedly recognize only PRA by IHC and develop specific antibodies to both PR isoforms.
c. Develop novel reliable methods to quantify the PR protein isoform ratio.
d. Determine whether there is a change in the PR isoform ratio between primary tumors and their metastatic spreads.
e. Determine whether PRA-H tumors will become PRB-H in recurrence.
f. Further explore mechanisms to re-express PRA in PRB-H tumors.
g. Design PRB selective inhibitors.
h. Dissect the role of GR and AR in the interplay with PR isoforms to design combined therapies.
i. Evaluate PR isoforms in breast cancer biopsies of patients undergoing clinical trials involving progestins/antiprogestins.

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j. Better characterize metastatic tumor models to further evaluate the role of antiprogestins/progestins as therapeutic agents in tumor progression.

Declaration of interest

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

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