Estrogen and progesterone receptors have distinct roles in the establishment of the hyperplastic phenotype in PR-A transgenic mice

Marina Simian[1,2], Mina J Bissell[1], Mary Helen Barcellos-Hoff[1,3], Gopalan Shyamala[^]

[1]Life Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley,

CA 94720, United States.

[2]Current address: Research Area, Instituto de Oncologia “Angel H. Roffo”, Avda. San Martin

5481, Buenos Aires, C1417DTB, Argentina

[3]Current address: Department of Radiation Oncology, New York University Langone Medical

Center, 566 First Avenue, New York, NY 10016, United States.

Corresponding author: Marina Simian, marina.simian@galuzzi.com

[^] Deceased

Abstract

Introduction: Expression of the 'A' and 'B' forms of progesterone receptor (PR) in an appropriate ratio is critical for mammary development. Mammary glands of PR-A transgenic mice, carrying an additional 'A' form of PR as transgene, exhibit morphological features associated with the development of mammary tumors. Our objective was to determine the roles of estrogen (E) and progesterone (P) in the genesis of mammary hyperplasias/preneoplasias in PR-A transgenics.

Methods: We subjected PR-A mice to hormonal treatments and analyzed mammary glands for the presence of hyperplasias and used BrdU incorporation to measure proliferation. Quantitative image analysis was carried out to compare levels of LAP and TGFβ1 between PR-A and PR-B transgenics. Basement membrane disruption was examined by immunofluorescence and proteolytic activity by zymography.

Results: The hyperplastic phenotype of PR-A transgenics is inhibited by ovariectomy, and is reversed by treatment with E+P. Studies using the antiestrogen ICI 182,780 or antiprogestins RU486 or ZK 98,299 show that the increase in proliferation requires signaling through E/ER-α but is not sufficient to give rise to hyperplasias, whereas signaling through P/PR has little impact on proliferation but is essential for the manifestation of hyperplasias. Increased proliferation is correlated with decreased TGFβ1 activation in the PR-A transgenics. Analysis of basement membrane integrity showed loss of laminin-5, collagen III and IV in mammary glands of PR-A mice, which is restored by ovariectomy. Examination of matrix metalloproteases (MMPs) showed that total levels of MMP-2 correlate with the steady state levels of PR, and that areas of laminin-5 loss coincide with those of activation of MMP-2 in PR-A transgenics. Activation of

MMP-2 is dependent on treatment with E and P in ovariectomized wild type mice, but is achieved only by treatment with P in PR-A mice.

Conclusions: These data establish a link between hormonal response, proliferation, modulation of MMP activity and maintenance of basement membrane integrity that depend on a balance in the expression levels of PR-A and B isoforms. Notably, concomitant increased proliferation, due to inhibition of TGFβ1 activation, and loss of basement membrane integrity, via increased MMP-2 activity, appear to be prerequisites for the PR-A hyperplastic phenotype.

Introduction

Progesterone receptor (PR) belongs to the superfamily of steroid receptors and mediates the action of progesterone in its target tissues [1,2]. In both humans and rodents, progesterone promotes the proliferation of epithelial cells that accompanies each menstrual/estrous cycle and pregnancy. In normal mammary glands of adult human and rodent females, PR expression is restricted to the luminal epithelial cells of the duct [3]. Studies on PR-null mutant mice have revealed that PR is essential for progesterone-dependent proliferation of epithelial cells [4].

PR exists in two isoforms, the 'A' and 'B' forms, and the expression of these, in an appropriate ratio, is critical for normal mammary development [5]. As such, in transgenic mice carrying either additional A form of PR (PR-A transgenics) or the B form (PR-B transgenics) mammary development is abnormal [6,7]. In particular, mammary glands of PR-A transgenics are characterized by extensive lateral branching, ductal hyperplasia, a disorganized basement membrane (BM) and loss of cell-cell adhesion [6]. Studies using the molecular markers for transformation, as defined by Medina and colleagues [8], revealed that these mammary glands contained at least two distinct populations of transformed epithelial cells. The ducts with normal histology contain cells resembling immortalized cells, while hyperplasias consist of cells in later stages of transformation associated with early preneoplasias [9].

The development of cancer is also associated with disruption of tissue architecture. Branching morphogenesis in the mammary gland is the culmination of hormone mediated proliferation and extracellular matrix (ECM) remodeling, these are each in turn dependent on the production of growth factors and the balance between ECM production and degradation [10]. Once established, the mammary gland undergoes rounds of highly orchestrated proliferation and morphogenesis

during pregnancy and involution, yet without losing the fundamental patterning of the gland. In contrast, hyperplasia is defined as loss of this patterning and is considered to be a precursor to neoplasia.

It is well established that PR-A can modulate the activities of both estrogen receptor-α (ER-α) and PR [11,12]. Accordingly, either or both estrogen and progesterone action, resulting from overexpression of PR-A, may mediate the abnormal mammary phenotype of PR-A transgenics. To this end the objective of our present studies was to identify the respective roles of estrogen and progesterone in the genesis of mammary hyperplasias/preneoplasias in PR-A transgenic mice.

Materials and methods

Mice, treatment with steroids and tissue preparations

All mice used in these studies were of FVB strain. The mice were housed and cared for in accordance with the NIH guide to humane use of animals in research. All experiments were conducted with Lawrence Berkeley National Laboratory institutional review and approval. Animals were killed by CO_2 inhalation and cervical dislocation at the indicated times in accordance with AAALAC guidelines.

PR-A transgenic mice [6] and PR null mutant mice [4] have been described previously. Nulliparous adult (9-12 weeks old) mice were used either as is or ovariectomized and/or treated with steroids for indicated times. For zymography studies, prior to tissue collection, mice were perfused with 10 ml of PBS and the tissues were frozen in liquid nitrogen and stored at –70˚C until used.

For studies with steroids, nulliparous adult mice were used either as intact or after ovariectomy. Estradiol (1μg/mouse) and/or progesterone (1mg/mouse) were administered as described previously [3]. Antiestrogen, ICI 182,780 (50 μg/mouse, Tocris Cookson Inc., Ellisville, MO) or antiprogestin, mifepristone (RU486, Sigma 16 μg/g body weight) or ZK 98,299 (16 μg/g body weight, gift from Dr. Ming-Wei Wang) were administrated daily for four days. For cell proliferation studies, mice were administered 160 μg/g body weight of 5-bromo-2-deoxyuridine (BrdU, Sigma, St. Louis, MO) 2 hours prior to sacrifice.

For mammary wholemounts, one of number 4 inguinal mammary gland was fixed in Carnoy's solution and stained in Alum Carmine. For immunohistochemical analyses on paraffin sections,

mammary tissues were collected, fixed in 4.7% buffered formalin (Fisher Scientific, Pittsburgh, PA), dehydrated, embedded in paraffin and cut into 5 µm thick sections. For immunoflourescence analyses on frozen sections, the entire no. 4 inguinal mammary glands were mounted and quick-frozen in OCT. Cryostat sections (5-10µm thick) were cut and mounted onto glass slides and fixed for 2 minutes in methanol/acetone (1:1).

Antibodies

The antibodies used were: anti-BrdU, rat monoclonal antibody (Harlan Sera-Lab Ltd., Loughborough, UK); goat anti-LAP and chicken anti-transforming growth factor beta 1 (TGFβ1; R&D Systems, Minneapolis, MN); MMP-2 (Chemicon International, Inc.), Collagens I, III and IV (Southern Biotechnology Assoc.Inc.), laminin-1 (Telios Pharmaceuticals), laminin- 5 (a gift from Dr. V. Quaranta).

Immunohistochemistry and immunofluorescence

BrdU positive cells were analyzed in paraffin embedded sections as described previously [9]. Briefly, paraffin sections were deparaffinized and rehydrated prior to antigen retrieval and treatment with specified antibodies. The antigen-antibody complexes were identified using Universal DAKO LSAB2 labeled streptavidin-biotin peroxidase kit (DAKO). The sections were counterstained with Mayer's hematoxylin solution (DAKO). After counterstaining, nuclei negative for the antigen appeared purple-blue and positive nuclei appeared brown.

Immunofluorescence assays were performed as described previously [3]. Briefly, non-specific binding sites were blocked by incubation in a blocking buffer (PBS containing either 1% Casein or 15% FCS and 0.2% Tween 20), for 30 minutes at ambient temperature. Sections were then

treated with primary antibodies, washed with PBS and incubated with fluorescein (FITC) conjugated secondary antibodies (dissolved in the blocking buffer) for 30 minutes at ambient temperature. Slides were then washed five times with PBS and the nuclei were stained with 4',6-diamino-2-phenylindole (DAPI) and mounted with Vectashield (Vector Laboratories).

Image Acquisition and Processing

Immunofluorescence images were obtained using a 40X 0.75 numerical aperture Zeiss Neofluor objective on a Zeiss Axiovert equipped with epifluorescence. A multiband pass dichroic mirror, barrier filter and differential wavelength filter wheel combination was used to selectively excite fluorochromes in sequence. Images were captured using a scientific-grade 12-bit charged coupled device (KAF-1400, 1317x1035, 6.8 um square pixels) on Xillix digital camera (Vancouver, Canada). Relative intensity of images were maintained when constructing figures by using Scilimage (TNO Institute of Applied Physics, Delt, The Netherlands) to scale the 12 bit data to a common 8-bit scale using the data set minimum and maximum. False color was assigned accordingly. Internal standardization was achieved by comparing only images stained with the same antibodies in the same experiment, captured with identical parameters and scaled and displayed identically.

Zymography

Mammary glands were homogenized on ice in RIPA insoluble buffer (50mM Tris, pH 8.0 containing 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate and 1% NP40). The homogenates were centrifuged for 15 minutes at 4°C and the supernatants were subjected to electrophoresis on gelatin substrate gels (8.8% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels containing 1

mg/ml of gelatin), as described previously [13]. Subsequently, the gels were treated with 2.5% Triton X-100 for 30 min., followed by incubation for 24 hrs at 37°C in a buffer containing 100 mM Tris-HCl, pH 7.4, and 15 mM CaCl2. The gels were stained with Coomassie Blue R-250 and destained with water until clear zones indicative of proteolytic activity emerged against a blue background. The zymograms were scanned and subjected to densitometric analyses using the PC version of NIH image (Scion Corporation).

Statistics

The data are presented as means ± S.E.M. The differences between the various experimental groups were analyzed by Student's t test and were considered significant when $P < 0.05$ was obtained. A Kolmogorov-Smirnov Comparison of two data sets was used to compare the TGFβ1 and LAP data, with $P< 0.05$ considered significant.

Results

Mammary phenotype of PR-A transgenics is dependent on ovarian steroids

We previously showed that the mammary glands of adult PR-A transgenic mice are characterized by extensive lateral branching and the presence of very thick ducts [6]. Histological analyses revealed that the thickening of the duct walls was due to the presence of multiple layers of cells, in contrast to the monolayer, or simple epithelium found in ducts of wild type mice [6]. These hyperplasias were characterized by a disruption of the basement membrane and loss of cell-cell adhesion [6]. The morphology of the mammary glands of PR-A transgenics under different hormonal conditions is shown in Fig. 1. The excessive ductal side branching in glands of adult females (Fig.1, panel A) was abolished upon ovariectomy (Fig.1, panel B). Short term administration of estradiol alone initiated epithelial proliferation, characterized by the presence of numerous side buds (Fig.1, panel C); however, when progesterone was administered with estradiol, the effects of ovariectomy on the PR-A phenotype was fully reversed such that the hyperplastic mammary phenotype resembled that of intact mice (Fig.1, compare panels A and D).

To identify the relative importance of signaling through estrogen versus progesterone in eliciting the excessive ductal growth, intact mice were treated with either antiestrogen ICI 182,780 or antiprogestins RU486 or ZK 98,299. Surprisingly, in mammary glands of mice treated with ICI 182,780 the ductal growth was greatly diminished and was comparable to that of ovariectomy (Fig. 1, compare panels B and E). However, mammary glands of mice treated with ZK 98,299 or RU486 still exhibited side branching, similar to intact untreated mice (Fig. 1, panels, F and G respectively).

Quantitation of the gross histological appearance confirmed the absence of hyperplasias in mammary glands of ovariectomized PR-A transgenics or intact mice treated with ICI 182,780 and the prevalence of hyperplasias in mammary glands of intact mice treated with RU486 or ZK 98,299, which was similar to untreated intact mice (Fig. 2). Likewise, treatment of ovariectomized mice with estradiol alone, but not progesterone, led to a modest increase in the number of hyperplasias; however, upon treatment with both estradiol and progesterone their prevalence was equivalent to that seen in intact mice. These data suggest that E and P have functionally distinct roles in hyperplasia.

In parallel analyses for cell proliferation, BrdU positive cells were rarely detected in mammary glands of ovariectomized mice, but increased upon treatment with estradiol into the range seen in intact mice (Fig. 3). Similar to ovariectomy, BrdU positive cells were infrequent in intact mice treated with the antiestrogen ICI 182,780. However the number of BrdU positive cells in both normal and hyperplastic ducts of intact mice treated with ZK 98,299 was essentially similar to that of untreated intact mice (Fig. 3). Our earlier studies [9] had shown that antiprogestin, RU486, had no effect on cell proliferation in the hyperplasias.

These observations established that (a) the increase in cell proliferation, a pre-requisite for the genesis of hyperplasias, required signaling through estradiol/ER but was not sufficient to give rise to overt hyperplasias in magnitude corresponding to that seen in intact untreated PR-A mice, (b) while the overexpression of PR-A leads to the development of the hyperplasias, cross talk with ER is essential for the manifestation of the hyperplastic phenotype, and (c) antiprogestins ZK 98,299 or RU486 were not sufficient to revert the hyperplastic phenotype. On the whole, these data reveal that in the context of PR-A overexpression in the mouse mammary gland, the

requirement for estradiol and progesterone are mediated through different mechanisms that are however interdependent.

TGFβ1 expression is impeded in mammary glands of PR-A transgenic mice

In adult female mice, mammary epithelial cells are maintained in a quiescent state in part due to the actions of TGFβ1 [14]. TGFβ1 is produced as a latent complex with latency-associated peptide (LAP) and needs to be activated to exert its biological effect [15]. In the mammary gland, activation of TGFβ1 is regulated by estradiol and progesterone, and restricts the proliferative response to these steroids [14]. Therefore, it is plausible that the hyperproliferation of mammary epithelial cells in PR-A transgenics is due to diminished expression and/or activity of TGFβ1. One of a few tools available to assess TGFβ1 in situ is the double staining for LAP and TGFβ1 epitopes that are revealed upon activation [16]. Mammary glands of PR-A transgenics showed a statistically significant decrease in both LAP and TGFβ1 as compared to wild type litter mate controls (Fig. 4, panels A and B). In transgenic mice carrying additional B form of PR (PR-B transgenics) mammary development is abnormal in that they have a limited capacity for ductal elongation, in contrast to hyperplasias exhibited by PR-A transgenics [6,7]. To determine if reduced expression of TGFβ1 associated with excessive ductal growth in PR-A transgenics was specifically due to overexpression of PR-A, we examined the expression patterns of TGFβ1 in mammary glands of PR-B transgenics. Compared to mammary glands of PR-A transgenics, both latent and active TGFβ1 were present at a higher level in PR-B transgenics, although only the increase in LAP was statistically significant (Fig. 4C). Thus, down regulation of total TGFβ1 mediated by PR-A, in contrast to up-regulation by PR-B, likely

mimics the *Tgfb1* heterozygote phenotype of exaggerated response to steroid hormone mediated proliferation [17].

Modulation of matrix metalloproteinase (MMP) activity and basement membrane (BM) integrity by estrogen and progesterone

Side branching and alveolar morphogenesis requires remodeling of the basement membrane (BM) [18]. We had previously shown discontinuous laminin-1 staining in the mammary glands of PR-A transgenics indicating a loss of BM integrity [6]. We show here that immunostaining for laminin-5 and collagen IV was also discontinuous. Furthermore, there was decreased collagen III immunoreactivity in the stroma surrounding the regions with aberrant epithelial structures (Fig. 5, panel A). Importantly, ovariectomy restored the integrity of the BM as revealed by continuous laminin-1 staining in PR-A mice (Fig. 5, panel B), and this effect was reversed by administration of estrogen and progesterone (Fig.5, panel B). It thus appears that upregulation of PR-A leads to remodeling of the BM.

Remodeling of the extracellular matrix (ECM) is regulated mainly by matrix metalloproteinases (MMPs) the expression of which is under developmental regulation in the mammary gland [19]. Furthermore, targeted overexpression of MMP-3 (stromelysin-1) in mammary epithelium leads to increased branching morphogenesis [20], hyperplasia [21] and tumors [22]. Therefore, to assess whether the loss in BM integrity observed in the mammary glands of PR-A transgenics was due to altered expression of MMPs, we examined the latent and active forms of MMPs by gelatin zymography. As shown in Fig. 6, panel A, total tissue extracts of mammary glands derived from either wild type and PR-A mice revealed three distinct bands corresponding to latent MMP-9 (LMMP-9), latent MMP-2 (LMMP-2) and active MMP-2 (AMMP-2).

Quantitation of the levels of total MMP-2 (Fig.6, panel A) and MMP-9 (not shown) as determined by densitometry did not reveal a significant difference between the mammary glands of wild type mice and PR-A transgenics. To further investigate whether there could be a link between the total levels of PR and MMP activity we analyzed tissue extracts derived from PR null mutant mice and compared them to their littermate controls. As shown in Fig.6, panel B, there was a statistically significant decrease in the levels of total MMP-2 in the PR null mutant mice, suggesting that the steady state levels of PR modulate proteolytic activity in the mouse mammary gland.

To determine whether signaling through ER and/or PR were involved in modulating MMP activities, we analyzed tissue extracts of ovariectomized mice exposed to exogenous estrogen and/or progesterone (Fig. 6, panel C). Mammary glands of wild type mice treated with estrogen and progesterone exhibited statistically significant increased levels of active MMP-2 (Fig. 6, panel C). This did not occur in the glands derived from animals treated with either estrogen or progesterone alone. As with wild type mice, MMP-2 activation was observed in mammary glands of ovariectomized PR-A mice treated with estrogen and progesterone; again estrogen alone did not have any effect on the levels of active MMP-2 (not shown); however, progesterone alone elicited MMP-2 activation in PR-A transgenics (Fig. 6, panel D).

To detect MMP activity within the tissue it is necessary to use a tool that can distinguish between the latent and active forms. We used an antibody that specifically detected latent MMP-2 and does not cross react with its active form. We correlated the localization of LMMP-2 to that of laminin-5 staining, which has been previously shown to be degraded by MMP-2 [23]. In mammary glands of wild type mice, and in regions where the ducts appeared normal in PR-A

transgenics, LMMP-2 and laminin-5 were detected in the periepithelial stroma (Fig. 7, panel A). However, in regions with aberrant structures, LMMP-2 was either reduced or dramatically absent and laminin-5 was essentially degraded. These observations that hyperplasias in the mammary glands of PR-A transgenics have increased MMP activity and increased BM degradation support the hypothesis that PR-A drives MMP-2 activation.

Finally, to exclude the possibility that the degradation of the BM was specifically due to overexpression of PR-A and was not the result of the overall imbalance in the ratio of the two isoforms of PR, we carried out laminin-1 stainings in mammary glands of PR-B mice. As shown in Fig. 7, panel B, the basement membrane was intact in PR-B mice.

Discussion

We have previously shown that mammary gland development in PR-A transgenic mice is abnormal, characterized by extensive ductal growth, lateral branching and loss of basement membrane integrity and cell-cell adhesion [6]. In this report we show that the proliferative phenotype of PR-A mice is dependent on ovarian steroids, where hyperplasia is mediated by distinct mechanisms. PR-A transgenic expression augments ER-α mediated proliferation while PR-A the degradation of the basement membrane, both essential requirements for the development of hyperplasias. Moreover, even though progesterone is required for the manifestation of the hyperplasias, antiprogestins have no effect on the established hyperplastic phenotype.

Signaling through estradiol/ER is required for proliferation, but is not enough to give rise to overt hyperplasias. Once established, these hyperplasias are dependent on ER signaling, as treatment with the antiestrogen ICI 182,780 abolishes them; we have not determined however, the relative role of ER-α and ER-β. On the other hand, the overexpression of PR-A leads to antiprogestin resistance, as neither ZK 98,299 nor RU486 had any effect on the expression of the hyperplasia in the intact mice. Both antiprogestins have similar mechanisms of action inducing PR binding to the DNA, but causing conformational changes that block co-activator binding to the receptor and thus make it transcriptionally inactive [24]. However, the antagonistic activity in both cases has been shown to depend on the cellular context that determines the degree of the antagonist/agonist activity of these compounds, which relies on the balance between expression and availability of coactivators and corepressors and the context of specific target promoters available in any given cell type [24]. Furthermore it is clear from the ovariectomized mice that

progesterone is necessary to induce the full phenotype. Thus, overexpression of PR-A in the mouse mammary gland seems to generate a cellular context that promotes hyperplasia, but whose maintenance is not dependent upon progesterone signaling per se.

The hyperproliferation observed in the mammary glands of PR-A transgenic mice may be due to the diminished expression found for both latent and active TGFβ1. Activation of TGFβ1 is regulated by estradiol and progesterone, which in turn restricts the proliferative response to these steroids [14]. Our studies suggest that physiological estradiol drives inhibition of TGFβ1 that in turn increases proliferation in response to estradiol, which is consistent with its action in estrus shown using TGFβ1 heterozygote mice [17]. Notably, the mammary glands of *Tgfb1* heterozygote mice are 2-4 times more proliferative yet there is no evidence of hyperplasia [17]. Thus, while similar to *Tgfb1* in regards to decreased TGFβ1 levels and increased proliferative response to steroid hormones, an additional action of PR-A must be necessary for the expression of hyperplasia. Since our studies show that signaling through progesterone has no impact on proliferation in hyperplastic PR-A mice but is required for the manifestation of the hyperplasias, we postulated that another effect must be required. We show that signaling through PR modulates the activation of MMP-2, and that areas of BM disruption coincide with loss of latent MMP-2. The integrity of BMs is dictated by the composition of MMPs, their inhibitors and the status of their activation [25]. MMPs have been shown to be key regulators of branching morphogenesis in the mouse mammary gland [20,26,27]. On the other hand it has long been known that both estrogen and progesterone affect mammary gland development regulating ductal elongation, side branching and differentiation. The development of PR and ER transgenic and knock-out mice in the last decade has provided additional evidence that confirm these results

[4,6,28]. However, to date there is insufficient evidence to address the intriguing question as to how hormonal action, MMP production and activation are linked in the mouse mammary gland.

We had previously shown that there was loss of laminin-1 in areas of aberrant epithelial morphology in PR-A transgenic mice [6]. We report now that in addition to laminin-1, immunoreactivity of laminin-5 and collagens IV and III are also lost. Thus, in the mammary glands of PR-A transgenics, the concurrent loss of laminin and collagen indicate that the ECM is broadly affected. More importantly the fact that the phenotype was reversible by ovariectomy points to a role for hormonal action in the maintenance of ECM integrity, which is supported by our observation that the administration of estradiol and progesterone to ovariectomized PR-A transgenic mice led to BM degradation, strongly supporting this notion.

Alterations in the mammary gland ECM have been previously described in transgenic mice expressing an active form of MMP-3 in the epithelium [21]. The fact that total MMP-2 levels in extracts derived from PR-A transgenic mice were not significantly different could be due to the heterogeneous phenotype, as previously shown [6], and is consistent with the statistically significant decrease we observed in MMP-2 levels only in PR null mutant mice. Moreover, immunolocalization studies support the notion of restricted phenotype as we found decreased LMMP-2 only in hyperplastic regions where laminin-5 was absent.

The finding that treatment of ovariectomized wild type mice with estradiol and progesterone leads to the activation of MMP-2 is the first direct demonstration that hormonal action modulates MMP activity in the mouse mammary gland. This result suggests an important role for MMP-2 during proliferation and differentiation that takes place during pregnancy. It is significant to note that while in wild type ovariectomized mice progesterone alone had no effect on MMP-2

activation (because there is little or no PR-A), it caused an increase in the activation of MMP-2 in mammary glands of ovariectomized PR-A transgenics, because they still expressed a high level of PR-A. Thus, in wild type mice ER would be necessary to support the levels of PR, but signaling through PR-A would be important for the activation of MMP-2. Using MMP-2 knock-out mice, Wiseman and collaborators have shown that during puberty MMP-2 positively regulates invasion of terminal end buds but that it inhibits side branching from mature ducts. They also postulated TGFβ1 as the candidate for the inhibitory action of MMP-2 on lateral branching at puberty [26]. This speculation is supported by recent modeling studies that demonstrate the effect of TGFβ1 as a branching inhibitor [29]. In mammary glands of PR-A transgenics there is a positive relationship between the steroid hormone dependent increase in MMP-2 activity and lateral branching, accompanied by a reduction in both the latent and active forms of TGFβ1. It is possible, however, that other proteases are also involved given that, although statistically significant, the degree of activation of MMP-2 was modest both in the wild type and in the PR-A transgenics. Given that MMP-2 is expressed by the periductal stroma and weakly by the adipose tissue [26] and that PR is expressed exclusively in the epithelial compartment [30], cross talk between both compartments seems to be a necessary condition for MMP-2 activation. A possible mediator of this cross talk may be MT1-MMP which is expressed both by the stroma and the epithelium [26]. This is supported by the fact that we have detected an increase in the levels of MT1-MMP in the mammary glands of pregnant mice and in certain areas in mammary glands of PR-A transgenic mice MT1-MMP levels were high (Simian et al, unpublished observations). Additionally, degradation of collagen IV appears to require the action of MT1-MMP [31]. It remains to be determined if PR positive cells are also MT1-MMP positive.

Conclusions

An imbalance in the relative ratio of PR-A and B isoforms has been observed in certain human mammary tumors, and this is often associated with overexpression of PR 'A' form [32-34]. Our studies show that this imbalance has a direct impact on three major phenomena underlying mammary biology i.e. steroid hormone responsiveness, TGFβ1 production, and ECM remodeling mediated by MMP activity, all of which are implicated in mammary epithelial cell transformation.

Abbreviations

PR= progesterone receptor; PR-A transgenics= mice carrying additional A form of PR; PR-B transgenics: mice carrying additional B form of PR; BM= basement membrane; ER-α= estrogen receptor-α; BrdU= 5-bromo-2-deoxyuridine; FITC=fluorescein; DAPI= 4',6-diamino-2-phenylindole; TGFβ1: transforming growth factor beta 1; LAP= latency associated peptide; MMP= matrix metalloproteinase; LMMP-9= latent MMP-9; LMMP-2= latent MMP-2; AMMP-2= active MMP-2; Ovx= ovariectomized.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MS performed the zymography studies, prepared the results and contributed to the drafting of the manuscript. MJB participated in the conception of the study, the direction of the research and the correction of the manuscript. MHBH participated in the conception of the study, carried out the TGFβ1 studies, the immunofluorescence of the basement membrane components and corrected the manuscript. GS conceived the study, directed the research, analyzed the data, kept the transgenic mice, carried out the whole mounts and BrdU studies, and drafted and corrected the manuscript.

Acknowledgments

Grant support: National Institutes of Health (National Institute of Aging grant RO1 AG022413) to MHBH and GS. MJB's laboratory is supported by grants from the U.S. Department of Energy, OBER Office of Biological and Environmental Research (DE-AC02-05CH1123), a Distinguished Fellow Award and Low Dose Radiation Program and the Office of Health and Environmental Research, Health Effects Division, (03-76SF00098); by National Cancer Institute awards 5 R01CA064786, R01CA057621, U54CA126552 and U54CA112970; by U.S. Department of Defense (W81XWH0810736). MS is supported by a Susan G. Komen for the Cure Foundation award (BCTR 0600341) and CONICET.

Reference List

1. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM: **The nuclear receptor superfamily: the second decade.** *Cell* 1995, **83:** 835-9.

2. Tsai MJ, O'Malley BW: **Molecular mechanisms of action of steroid/thyroid receptor superfamily members.** *Annu Rev Biochem* 1994, **63:** 451-86.

3. Shyamala G, Barcellos-Hoff MH, Toft D, Yang X: **In situ localization of progesterone receptors in normal mouse mammary glands: absence of receptors in the connective and adipose stroma and a heterogeneous distribution in the epithelium.** *J Steroid Biochem Mol Biol* 1997, **63:** 251-9.

4. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA Jr, Shyamala G, Conneely OM, O'Malley BW: **Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities.** *Genes Dev* 1995, **9:** 2266-78.

5. Shyamala G, Louie SG, Camarillo IG, Talamantes F: **The progesterone receptor and its isoforms in mammary development.** *Mol Genet Metab* 1999, **68:** 182-90.

6. Shyamala G, Yang X, Silberstein G, Barcellos-Hoff MH, Dale E: **Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor**

exhibit developmental abnormalities in mammary glands. *Proc Natl Acad Sci U S A* 1998, **95:** 696-701.

7. Shyamala G, Yang X, Cardiff RD, Dale E: **Impact of progesterone receptor on cell-fate decisions during mammary gland development.** *Proc Natl Acad Sci U S A* 2000, **97:** 3044-9.

8. Medina D: **Biological and molecular characteristics of the premalignant mouse mammary gland.** *Biochim Biophys Acta* 2002, **1603:** 1-9.

9. Chou YC, Uehara N, Lowry JR, Shyamala G: **Mammary epithelial cells of PR-A transgenic mice exhibit distinct alterations in gene expression and growth potential associated with transformation.** *Carcinogenesis* 2003, **24:** 403-409.

10. Fata JE, Werb Z, Bissell MJ: **Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes.** *Breast Cancer Res* 2004, **6:** 1-11.

11. Katzenellenbogen BS: **Mechanisms of action and cross-talk between estrogen receptor and progesterone receptor pathways.** *J Soc Gynecol Investig* 2000, **7:** S33-S37.

12. Kraus WL, Weis KE, Katzenellenbogen BS: **Inhibitory cross-talk between steroid hormone receptors: differential targeting of estrogen receptor in the repression of its transcriptional activity by agonist- and antagonist-occupied progestin receptors.** *Mol Cell Biol* 1995, **15:** 1847-1857.

13. Talhouk RS, Chin JR, Unemori EN, Werb Z, Bissell MJ: **Proteinases of the mammary gland: developmental regulation in vivo and vectorial secretion in culture.** *Development* 1991, **112:** 439-49.

14. Ewan KB, Oketch-Rabah HA, Ravani SA, Shyamala G, Moses HL, Barcellos-Hoff MH: **Proliferation of estrogen receptor-alpha-positive mammary epithelial cells is restrained by transforming growth factor-beta1 in adult mice.** *Am J Pathol* 2005, **167:** 409-417.

15. Shi Y, Massague J: **Mechanisms of TGF-beta signaling from cell membrane to the nucleus.** *Cell* 2003, **113:** 685-700.

16. Barcellos-Hoff MH, Ehrhart EJ, Kalia M, Jirtle R, Flanders K, Tsang ML: **Immunohistochemical detection of active transforming growth factor-beta in situ using engineered tissue.** *Am J Pathol* 1995, **147:** 1228-1237.

17. Ewan KB, Shyamala G, Ravani SA, Tang Y, Akhurst R, Wakefield L, Barcellos-Hoff MH: **Latent transforming growth factor-beta activation in mammary gland: regulation by ovarian hormones affects ductal and alveolar proliferation.** *Am J Pathol* 2002, **160:** 2081-2093.

18. Williams JM, Daniel CW: **Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis.** *Dev Biol* 1983, **97:** 274-90.

19. Talhouk RS, Bissell MJ, Werb Z: **Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution.** *J Cell Biol* 1992, **118:** 1271-82.

20. Sympson CJ, Talhouk RS, Alexander CM, Chin JR, Clift SM, Bissell MJ, Werb Z: **Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression [published erratum appears in J Cell Biol 1996 Feb;132(4):following 752].** *J Cell Biol* 1994, **125:** 681-93.

21. Thomasset N, Lochter A, Sympson CJ, Lund LR, Williams DR, Behrendtsen O, Werb Z, Bissell MJ: **Expression of autoactivated stromelysin-1 in mammary glands of transgenic mice leads to a reactive stroma during early development.** *Am J Pathol* 1998, **153:** 457-67.

22. Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z: **The Stromal Proteinase MMP3/Stromelysin-1 Promotes Mammary Carcinogenesis.** *Cell* 1999, **98:** 137-146.

23. Koshikawa N, Giannelli G, Cirulli V, Miyazaki K, Quaranta V: **Role of Cell Surface Metalloprotease MT1-MMP in Epithelial Cell Migration over Laminin-5.** *J Cell Biol* 2000, **148:** 615-624.

24. Leonhardt SA, Edwards DP: **Mechanism of action of progesterone antagonists.** *Exp Biol Med (Maywood)* 2002, **227:** 969-980.

25. Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N: **Metalloproteinases: role in breast carcinogenesis, invasion and metastasis.** *Breast Cancer Res* 2000, **2:** 252-257.

26. Wiseman BS, Sternlicht MD, Lund LR, Alexander CM, Mott J, Bissell MJ, Soloway P, Itohara S, Werb Z: **Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis.** *J Cell Biol* 2003, **162:** 1123-1133.

27. Witty JP, Wright JH, Matrisian LM: **Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development.** *Mol Biol Cell* 1995, **6:** 1287-303.

28. Bocchinfuso WP, Korach KS: **Mammary gland development and tumorigenesis in estrogen receptor knockout mice.** *J Mammary Gland Biol Neoplasia* 1997, **2:** 323-334.

29. Nelson CM, Vanduijn MM, Inman JL, Fletcher DA, Bissell MJ: **Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures.** *Science* 2006, **314:** 298-300.

30. Shyamala G, Chou YC, Louie SG, Guzman RC, Smith GH, Nandi S: **Cellular expression of estrogen and progesterone receptors in mammary glands: regulation by hormones, development and aging.** *J Steroid Biochem Mol Biol* 2002, **80:** 137-148.

31. Rowe RG, Weiss SJ: **Breaching the basement membrane: who, when and how?** *Trends in Cell Biology* 2008, **18:** 560-574.

32. Graham JD, Yeates C, Balleine RL, Harvey SS, Milliken JS, Bilous AM, Clarke CL: **Characterization of Progesterone Receptor A and B Expression in Human Breast Cancer.** *Cancer Research* 1995, **55:** 5063-5068.

33. Mote PA, Johnston JF, Manninen T, Tuohimaa P, Clarke CL: **Detection of progesterone receptor forms A and B by immunohistochemical analysis.** *J Clin Pathol* 2001, **54:** 624-630.

34. Mote PA, Bartow S, Tran N, Clarke CL: **Loss of co-ordinate expression of progesterone receptors A and B is an early event in breast carcinogenesis.** *Breast Cancer Res Treat* 2002, **72:** 163-172.

Figure Legends

Figure 1: Wholemounts of mammary glands of adult PR-A transgenic mice.

Whole mounts of mammary glands derived from: A) Intact PR-A transgenic mice; B) Ovariectomized PR-A transgenic mice; C) Ovariectomized PR-A transgenic mice treated with estradiol 1 µg for 5 days; D) Ovariectomized PR-A transgenic mice treated with estradiol 1 µg and progesterone 1 mg for 5 days; E) Intact PR-A transgenic mice treated with ICI 182,780 for 4 days; F) Intact PR-A transgenic mice treated with ZK98, 299 for 4 days; G) Intact PR-A transgenic mice treated with RU486 for 4 days.

Figure 2: Effects of hormonal treatments on the prevalence of hyperplasias in the mammary glands of PR-A transgenic mice.

Five to six tissue sections, corresponding to each group, and representing mammary glands from two to three PR-A transgenic mice, were stained with H&E and a minimum of 100 ducts per section were counted. Each mammary gland from each mouse was analyzed in duplicate. The data is presented as percentage of total ducts and represent the mean +/- S. E. M. Ovariectomy led to a decrease in the % of hyperplasias (Intact vs Ovx: $P \leq 0.01$), which was partially recovered by administration of estradiol (Ovx vs Ovx+E: $P \leq 0.01$), and completely recovered by treatment with estradiol 1µg and progesterone 1 mg (Ovx vs Ovx+E+P: $P \leq 0.001$). On the other hand

treatment with ICI 182,780 also led to a decrease in the % of hyperplasias (Intact vs Intact + ICI 182,780: $P \leq 0.001$), but neither the antiprogestins RU486 nor ZK 98,299 had an effect.

Figure 3: BrdU labeling indices in mammary glands of PR-A transgenic mice under various hormonal status.

The percentage of BrdU-positive mammary epithelial cells of normal ducts (ND) and hyperplastic ducts (HD) of PR-A transgenics under various hormonal statuses was analyzed. The data is presented as the mean +/- S.E.M. Mammary glands from a minimum of three mice were examined and mammary glands for each mouse were analyzed in triplicate. In each experiment, the percentage of immuno-positive cells was obtained by counting a minimum of 500 cells per gland.

Figure 4: TGFβ1 and LAP in mammary glands of PR-A and PR-B mice.

A) Examples of dual immunofluorescence staining to LAP (green) and TGFβ1 (red) in mammary glands derived from wild type and PR-A transgenic mice; both were decreased in the transgenic mice. Nuclei were counterstained with DAPI. B) Quantitative image analysis of the intensity of LAP and TGFβ1 immunoreactivity per cell in mammary glands of PR-A mice. Both LAP and TGFβ1 had a lower intensity in cells derived from PR-A transgenic mice as compared to wild type controls ($P < 0.001$ in both cases). C) Quantitative image analysis of the intensity of LAP and TGFβ1 immunoreactivity per cell in PR-A vs PR-B mice. Only the increase in LAP in

PR-B compared to PR-A mice was statistically significant ($P< 0.001$). The difference in active TGFβ1, although increased in PR-B mice, was not statistically significant.

Figure 5: Basement membrane disruption in mammary glands of PR-A transgenic mice: regulation by ovarian hormones.

A) Laminin-5, collagen IV and collagen III immunoreactivity (green) circumscribed the mammary epithelium of wild type mice, but were decreased in the glands of PR-A transgenic mice. Nuclei were counterstained with DAPI (blue). B) Ovariectomy of PR-A mice led to a recovery of the basement membrane integrity as shown by the continuous laminin staining. However, upon treatment with estrogen 1 μg and progesterone 1 mg for four days, the staining was lost.

Figure 6: Analyses for MMPs in mammary glands of adult wild type and PR-A transgenic mice by zymography.

A) Zymogram of protein extracts prepared from mammary glands of two PR-A mice, and a litter mate control. The graph shows the comparison of the levels, estimated by laser densitometry, of total MMP-2 in transgenic (n=5) and littermate control (n=3) mice. No statistically significant differences were found. Similar results were obtained for MMP-9 (not shown). B) Zymogram of protein extracts prepared from mammary glands of two PR null mutant mice (PR-/-), and a litter mate control. The graph shows the comparison of the levels, estimated by laser densitometry, of

total MMP-2 in PR-/- (n=5) and littermate control (n=4) mice. There was a statistically significant reduction in the total levels of MMP-2 in the PR null mutant mice ($P \leq 0.01$). C) Representative zymograms showing activation of MMP-2 in wild type mice only when they were treated with estrogen+progesterone (E+P). Representation of mean ± S.E.M. corresponding to the densitometric values of active MMP-2 ovariectomized (ovx, n=6), ovariectomized treated with E (ovx+E, n=4), ovariectomized treated with P (ovx+P, n=3) and ovariectomized treated with E+ P (ovxE+P, n=3) mice. In all cases values were normalized to the controls that were considered equal to 1. The increase in the levels of active MMP-2 were statistically significant in the ovxE+P group compared to ovx ($P \leq 0.001$), ovx+E ($P \leq 0.05$) and ovx+P ($P \leq 0.01$). D) Zymogram and densitometric analysis of mean ± S.E.M. showing activation of MMP-2 by progesterone in ovariectomized PR-A mice (Ovx+P, n=2) compared to ovariectomized mice (ovx, n=2). The increase in AMMP-2 activity was statistically significant ($P \leq 0.05$). For all graphs, "n" represents the number of glands analyzed from different mice.

Figure 7: In situ localization of MMP-2 and laminin-5 in mammary glands of adult wildtype and PR-A transgenics, and laminin staining in PR-B transgenics.

A) MMP-2 and laminin-5 were detected in fixed frozen sections of mammary glands, using an indirect immunofluorescence assay, as described in text. Blue represents DAPI staining of the nuclei, green represents FITC staining corresponding to immunoreactive latent MMP-2 and red represents Texas Red staining corresponding to immunoreactive laminin-5. Staining in glands from wild type mice and in normal structures derived from PR-A mice showed continuous laminin-5 staining and presence of MMP-2 in the periepithelial stroma. However, in aberrant

structures derived from glands of PR-A transgenic mice, staining for both laminin-5 and latent MMP-2 were lost (right panels). Top left panel, a mammary duct from wild type mouse; bottom left panel: a "normal" duct from a PR-A transgenic; top right panel: a duct from a PR-A transgenic containing both "normal" and morphogenetically disturbed epithelium; bottom left panel: an aberrant structure in a PR-A transgenic. B) Laminin staining (green) in a mammary gland derived from a PR-B transgenic mouse shows that is circumscribes the epithelium as in the wild type control. Nuclei were counterstained with DAPI (blue).

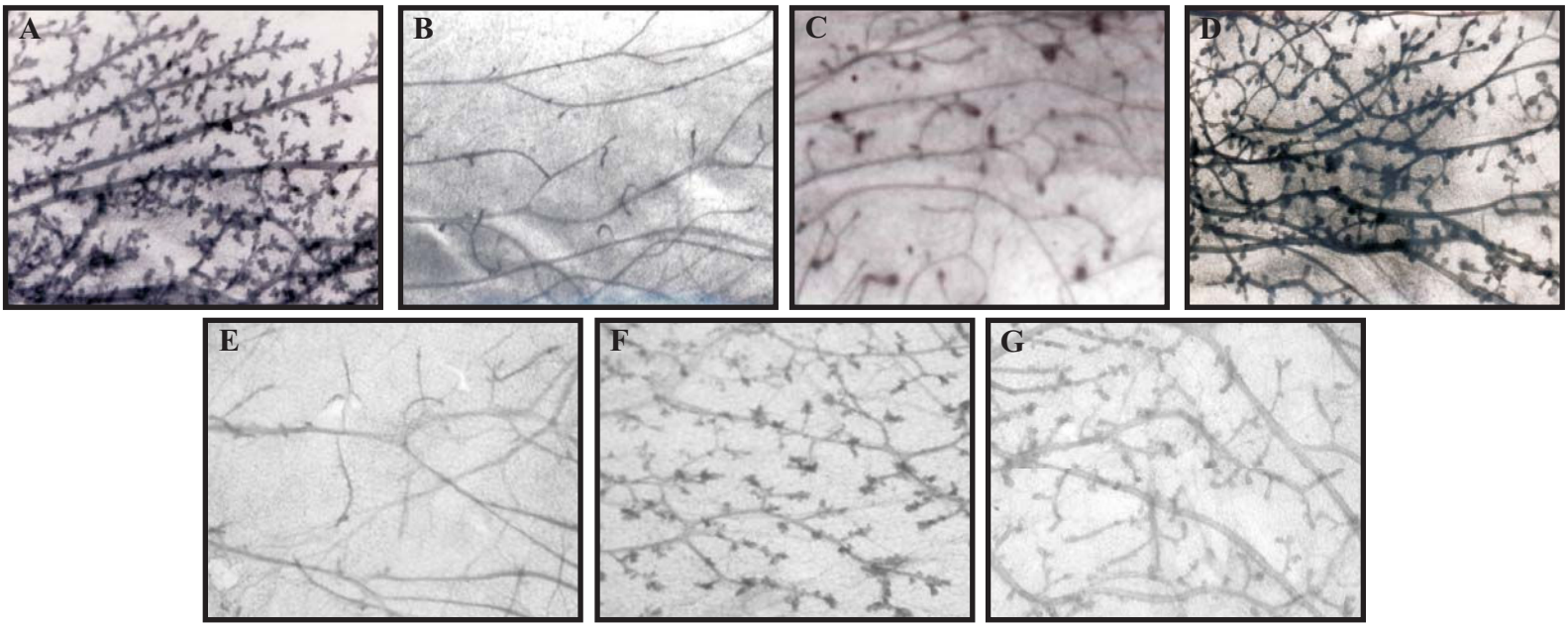


Figure 1

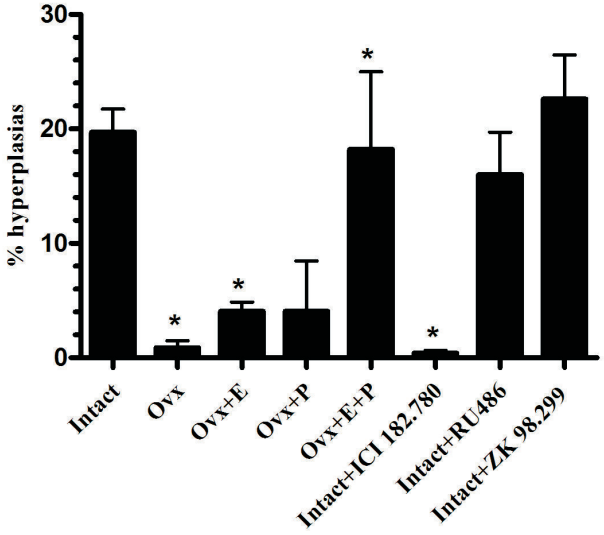

% hyperplasias
30
20
10
0
*
*
*
*
Intact
Ovx
Ovx+E
Ovx+P
Ovx+E+P
Intact+ICI 182.780
Intact+RU486
Intact+ZK 98.299

Figure 2

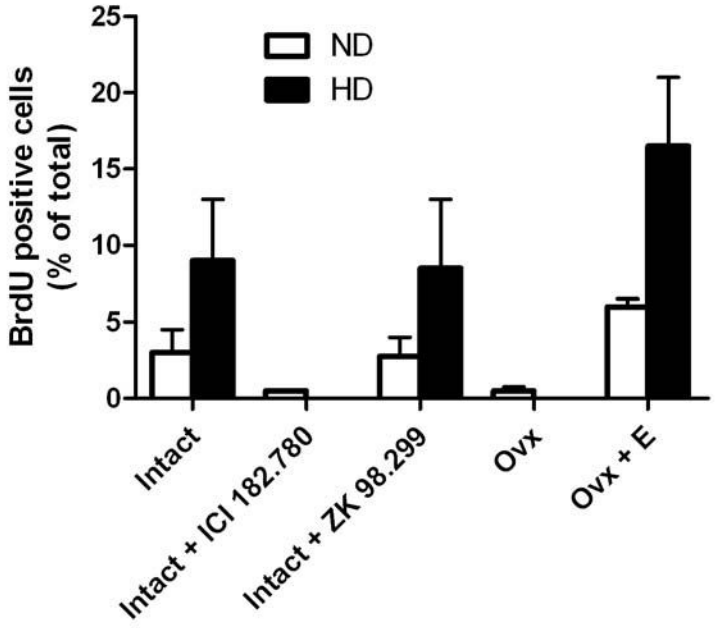
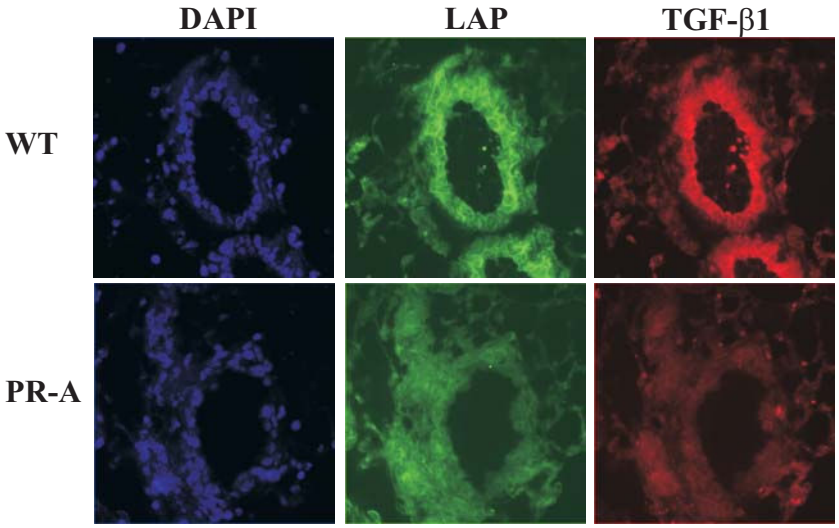
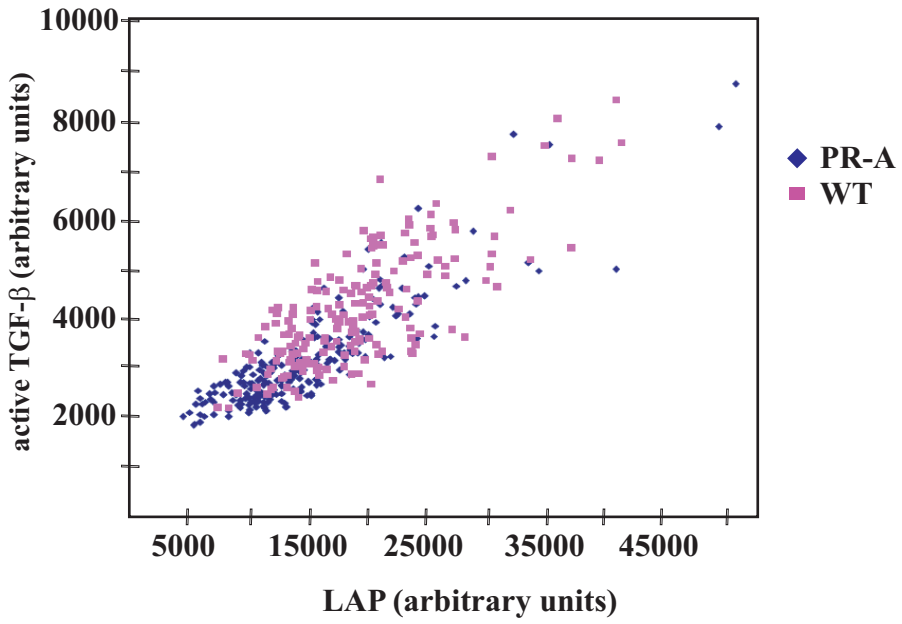

BrdU positive cells
(% of total)
25
20
15
10
5
0
ND
HD
Intact
Intact + ICI 182.780
Intact + ZK 98.299
Ovx
Ovx + E

Figure 3

A


DAPI
LAP
TGF-β1
WT
PR-A

B


10000
8000
6000
4000
2000
active TGF-β (arbitrary units)
5000
15000
25000
35000
45000
LAP (arbitrary units)
PR-A
WT

C

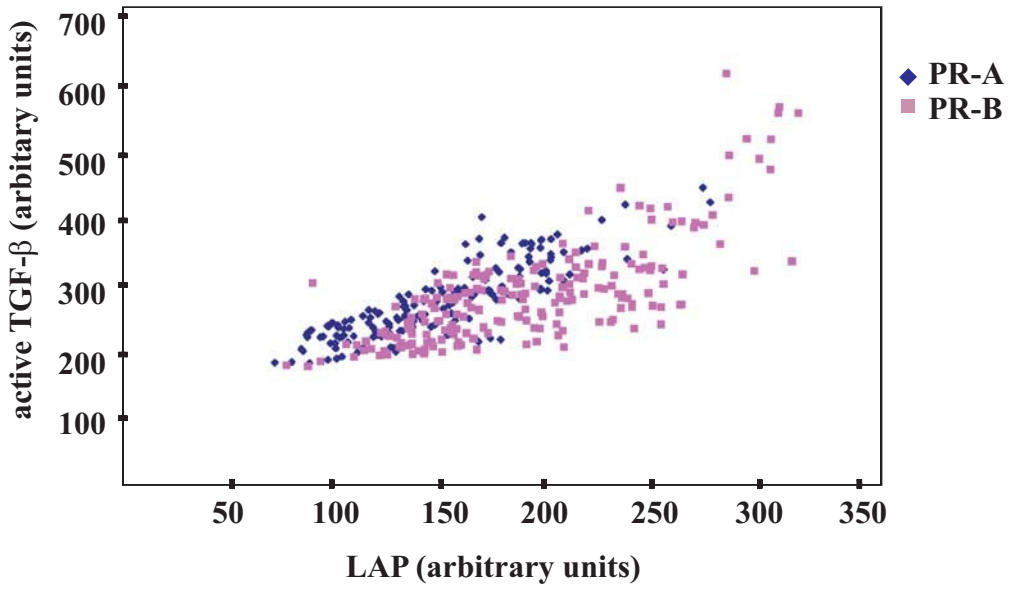

700
600
500
400
300
200
100
active TGF-β (arbitary units)
50
100
150
200
250
300
350
LAP (arbitrary units)
PR-A
PR-B

Figure 4

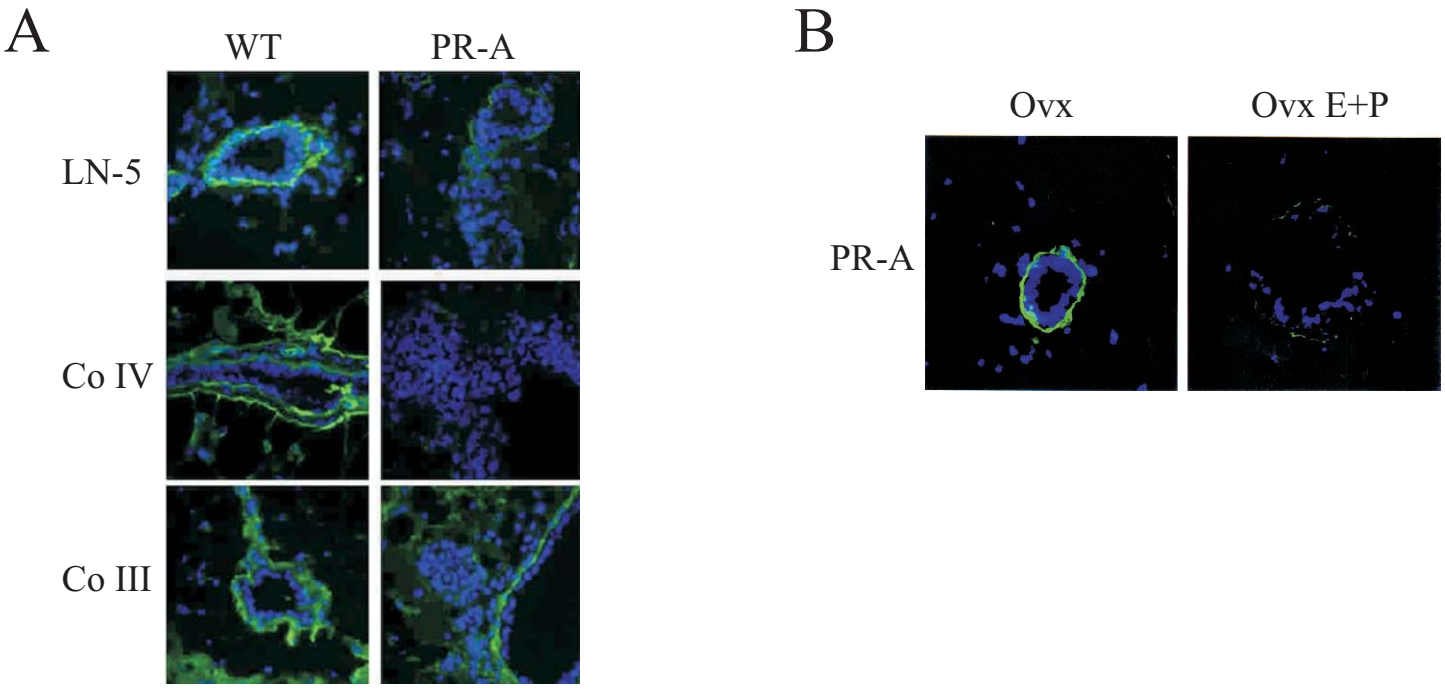


Figure 5

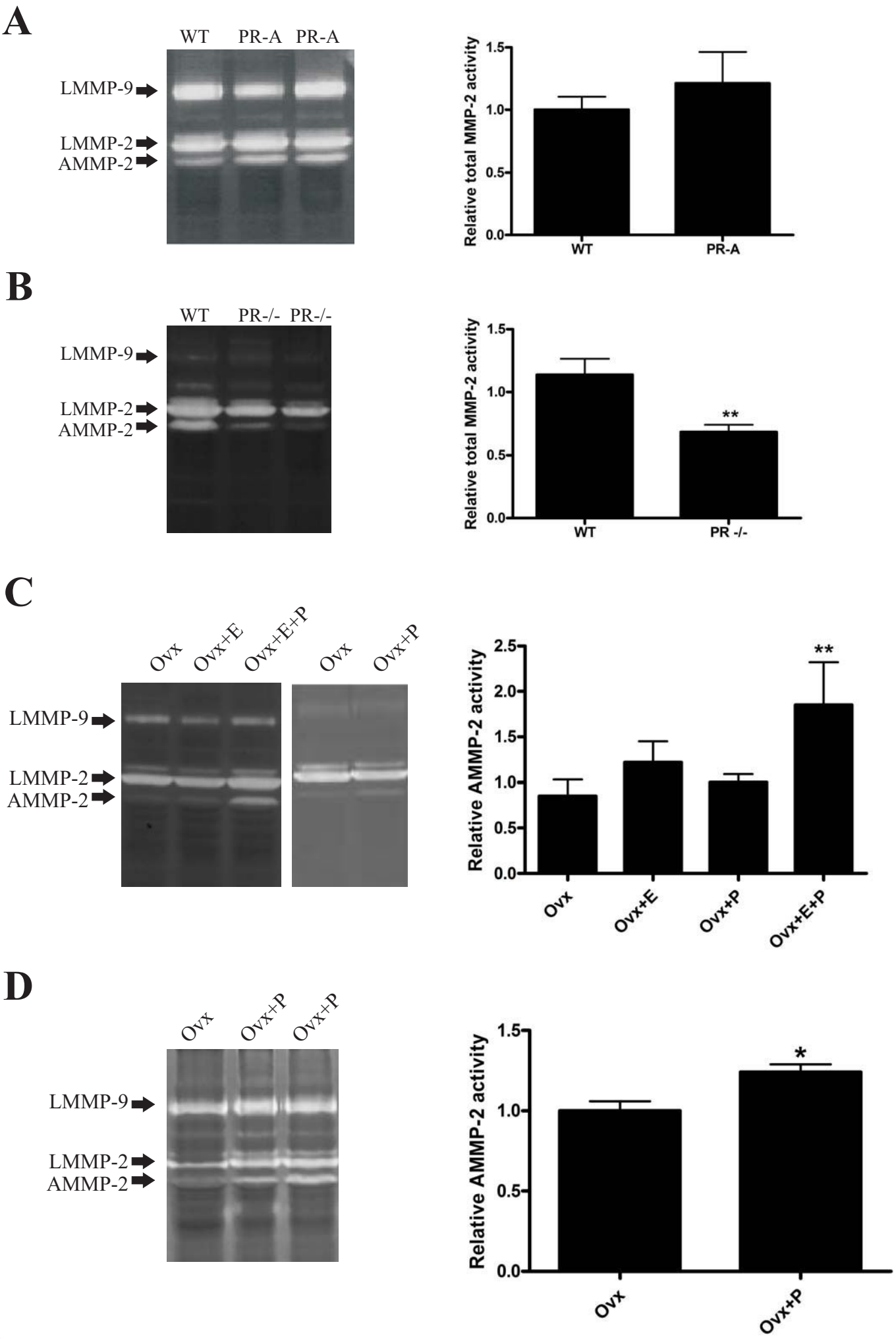

A
WT
PR-A
PR-A
LMMP-9
LMMP-2
AMMP-2
Relative total MMP-2 activity
1.5
1.0
0.5
0.0
WT
PR-A
B
WT
PR-/-
PR-/-
LMMP-9
LMMP-2
AMMP-2
Relative total MMP-2 activity
1.5
1.0
0.5
0.0
**
WT
PR -/-
C
Ovx
Ovx+E
Ovx+E+P
Ovx
Ovx+P
LMMP-9
LMMP-2
AMMP-2
Relative AMMP-2 activity
2.5
2.0
1.5
1.0
0.5
0.0
**
Ovx
Ovx+E
Ovx+P
Ovx+E+P
D
Ovx
Ovx+P
Ovx+P
LMMP-9
LMMP-2
AMMP-2
Relative AMMP-2 activity
1.5
1.0
0.5
0.0
*
Ovx
Ovx+P

Figure 6

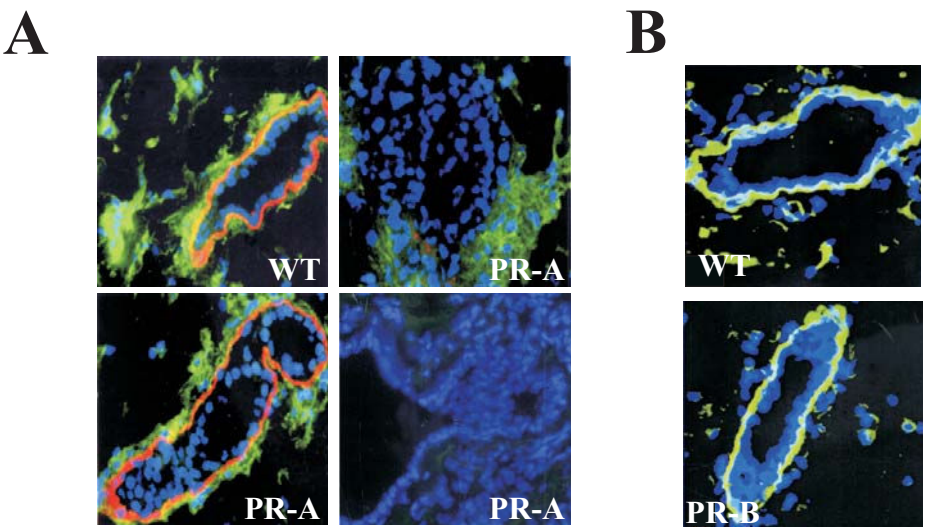


Figure 7