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Original contribution

Tissue-specific quantification and localization of androgen and estrogen receptors in prostate cancer☆,☆☆

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Summary Androgens and estrogens, working together, promote prostate cancer (PRCA) initiation and progression, with androgens acting via androgen receptor (AR) and estrogens acting primarily through estrogen receptor α (ERα). While the interplay between these steroid hormones has been established, the interaction between steroid hormone receptors in prostatic disease remains unstudied. The goal of this study was to objectively determine the incidence, stage specificity, and tissue/cell type specificity of AR and ERα expression, both independently and simultaneously, during the progression of PRCA. Using multiplexed immunohistochemistry and multispectral imaging analysis, AR, ERα, and smooth muscle α-actin expression was detected and quantitated in benign prostate tissue (BPT), high-grade prostatic intraepithelial neoplasia (HGPIN), PRCA, and metastasis (MET) from patient specimens (n = 340). Epithelial AR expression was significantly increased in HGPIN, PRCA, and MET compared with BPT, whereas ERα expression in epithelial and stromal cells was highest in HGPIN. With analysis of AR and ERα coexpression, we identified a unique population of double-positive ($AR^+/ER\alpha^+$) cells that

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increased in HGPIN specimens in both the stroma and the epithelium. Double-negative ($AR^-/ER\alpha^-$) cells significantly decreased across PRCA progression, from 65% in BPT to 30% in MET. Preliminary analysis of this $AR^+/ER\alpha^+$ population indicates potential cell type specificity in smooth muscle α-actin–negative stromal cells. This study demonstrates stage-, tissue-, and cell type–specific AR and ERα expression changes during PRCA progression, both independently and coexpressed. A more complete understanding of steroid hormones and their receptors in the initiation and progression of prostatic disease may elucidate improved strategies for PRCA prevention or therapy.

1. Introduction

In 2019, an estimated 174 650 men in the United States will be diagnosed as having prostate cancer (PRCA), and nearly 32 000 men will die as a result [1]. Because PRCA is the most prevalent cancer diagnosed among men in the United States, understanding the molecular changes associated with disease initiation and progression is critical for better diagnosis and treatment of men with prostatic disease. For decades, it has been known that sex steroid hormones and their receptors play an important role in regulating the prostate, both in development and in disease [2-4]. Although the interplay between hormone receptors has recently been recognized [5-7], the incidence, disease stage specificity, and cell type specificity of this interaction remain unresolved.

Androgens, acting via the androgen receptor (AR), play key roles in normal and neoplastic prostate growth [8,9]. Although it is well accepted that AR is expressed in the nuclei of stromal and epithelial cells of the prostate [9,10], there are discrepancies among reports of AR expression in PRCA progression [11-13]. Estrogens have also been implicated in the development and progression of PRCA, although the role of estrogens in prostatic disease is not well understood [14-16]. Unlike androgens, estrogens work primarily through 2 cognate receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ). In the prostate, several lines of evidence support the concept that ERα promotes prostatic epithelial proliferation during carcinogenesis, whereas ERβ functions as a tumor suppressor [3,4,14,17]. Because ERβ is considered to be lost during PRCA progression [14,17], this study will focus on the role of ERα. Similar to AR, the literature on ERα expression during PRCA progression is inconsistent [12,18-20]. Although there is some evidence of clinical success in targeting ERα for PRCA therapy or prevention [21], the interaction between AR and ERα in this context remains unresolved.

Recently, there has been an increasing interest in the synergy between steroid hormone receptors and their combined role in PRCA development and progression. It has been established that both androgens and estrogens are required for malignant transformation of cells in early PRCA progression [9,22], suggesting that there may be receptor interaction during PRCA initiation. Others have identified a role for AR/ERα cooperation in androgen-targeted therapy resistance [5], where prostate tumor heterogeneity underlying therapy resistance can be overcome by cotreatments targeting both AR and ERα signaling. Alternatively, cells that express AR and ERα may be intrinsically resistant to adrogen deprivation therapy because of sustained growth signaling through an ERα-dependent mechanism. A recent study showed expression of canonical AR-target gene fusion TMPRSS-ERG is working through ERα, not AR [7]. However, another study suggests that AR and ERα colocalization in the nucleus occurred with estrogen, not androgen, stimulation, suggesting that adrogen deprivation therapy may not be sufficient to prevent AR nuclear translocation [6]. Although these data introduce a potential interplay between AR and ERα in PRCA initiation or progression, the incidence and stage specificity of this interaction have not yet been identified.

Because of the inconsistencies in published data regarding AR and ERα expression in the prostate and the lack of information regarding coexpression of these receptors, there has been difficulty in discerning their combined significance in PRCA progression. In this study, we used an automated pathology analysis platform to quantitatively assess protein expression and localization of AR and ERα during PRCA progression, both simultaneously and independently [23]. This objective machine learning–based approach minimizes the limitations of previous studies (eg, staining technique/interobserver variability) and enables analysis of AR and ERα coexpression and colocalization in the same cell [23]. In addition, multiplexed staining with smooth muscle α-actin (SMA) allows for preliminary analysis of cell type specificity of AR and ERα coexpression. With this technology, we aim to determine (1) the incidence of AR and ERα colocalization in human prostate, (2) the stage of prostatic disease at which AR and ERα are coexpressed, and (3) preliminary cell type specificity of AR and ERα coexpression. A more precise and quantitative understanding of AR and ERα localization and expression at different stages of PRCA could lead to a more complete picture of disease progression and potentially offer insight into new approaches for prevention or therapy.

2. Materials and methods

2.1. Immunohistochemistry

To assess AR and ERα protein localization and abundance during PRCA progression, we used a previously described tissue microarray composed of duplicate tissue cores from prostates of different disease stages [23]. In accordance with the institutional review board, informed consent was obtained for experimentation with human samples. Antibodies (ARs: Biocare Medical ACI-109-A, 1:50; ERα: Thermo Scientific RM-9101, 1:400) were validated during optimization (Supplementary Fig. S1). Tissue cores consisted of benign prostate tissue (BPT), not inclusive of BPH (n = 101 cores, 52 patients), high-grade prostatic intraepithelial neoplasia (HGPIN; n = 50 cores, 25 patients), primary tumor (PRCA; n = 141 cores, 73 patients), and metastatic tumors (MET; n = 44 cores, 22 patients) [23]. Multiplexed immunohistochemistry (IHC) was performed to detect AR, ERα, and SMA as previously described [23,24]. SMA was used to designate stroma for stromal versus epithelial analysis.

2.2. Image analysis

For automatic image acquisition, we used the Vectra platform (PerkinElmer, Waltham, MA) and InForm 1.4 software, (PerkinElmer) for image analysis [23]. Using InForm 1.4 software (PerkinElmer), 18% of the total images (rendering approximately 97% accuracy) were trained by a genitourinary pathologist (W. H.) to segment nucleus from cytoplasm and epithelium from stroma. This created an algorithm enabling automated cell and tissue segmentation for each tissue compartment within each core sample. The same algorithm and threshold was then applied to the entire tissue microarray.

2.3. Staining quantification

AR and ERα stainings were quantified as the percentage of positive nuclei divided by the total number of nuclei in the respective tissue compartment (stroma and epithelium). To account for cells or tissue not already identified, we established a third compartment designated as "other," which included artifact, edge effect, nerves, red blood cells, and inflammatory cells. Colocalization of AR and ERα, within each cell was assessed and used to quantify double-positive cells (AR and ERα) in each tissue compartment [24]. Using raw cell segmentation data exported from inForm v1.4 (PerkinElmer) and manual thresholding of AR (mean optical density [OD] threshold, 0.02), ERα (mean OD threshold, 0.125), and SMA (mean OD threshold, 0.08), we calculated the proportion of $ER\alpha^+/AR^+$ double-positive cells within the SMA-negative stromal compartment.

2.4. Statistical analysis

GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA) was used for statistical analysis. We assessed differences among continuous variables with 1-way analysis of variance. Tukey multiple comparison test was used to determine mean differences of BPT compared with HGPIN, PRCA, and MET. Data in bar graphs and tables show mean ± SEM. For all analyses, $P < .05$ was considered statistically significant.

3. Results

3.1. Tissue and cell segmentation

Using machine learning software, we objectively segmented tissue types (stroma, epithelia) and cell compart-

Table Cell quantification and tissue segmentation in PRCA

	BPT	HGPIN	PRCA	MET
No. cells				
Total	1153 ± 36	1097 ± 73	1498 ± 37 *	1827 ± 109 *
Epithelial	402 ± 29	604 ± 59	822 ± 46 *	1169 ± 117 *
Stromal	487 ± 23	331 ± 34 **	436 ± 25	375 ± 89
% of cells				
Epithelial	33 ± 1.9	52 ± 2.7 *	52 ± 2.1 *	62 ± 4.9 *
Stromal	43 ± 1.7	30 ± 2.5 ***	30 ± 1.8 *	20 ± 4.3 *
% Tissue area				
Epithelial	16 ± 1.2	17 ± 1.5	33 ± 1.7 *	44 ± 3.8 *
Stromal	35 ± 1.8	33 ± 3.0	29 ± 1.8	23 ± 4.5 ***

All values are presented as mean ± SEM per core.
* $P < .001$ compared with BPT.
** $P < .05$ compared with BPT.
*** $P < .01$ compared with BPT.

ments (nucleus, cytoplasm) across disease progression in human prostate tissue (n=384). With this separation, we quantified cell number (based on nuclei) within each tissue type across PRCA progression, as shown in Table. The total number of epithelial cells increased from BPT to PRCA and from BPT to MET ($P<.001$ for both), whereas the percentage of epithelial cells increased from BPT to HGPIN, PRCA, and MET ($P<.001$ for all). There was a concordant decrease in the number of stromal cells from BPT to HGPIN ($P<.05$), and the percentage of stromal cells decreased from BPT to HGPIN ($P<.01$), PRCA, and MET ($P<.001$ for both). As expected, the percent of tissue area increased for the epithelial compartment in PRCA and MET compared with BPT (P .001 for both), but decreased for the stromal compartment in MET versus BPT ($P<.01$). These segmentation techniques provide evidence of the sensitivity and accuracy of the software and serve as the basis for our analysis of AR and ERα positivity within the nucleus of stromal versus epithelial tissue.

3.2. AR tissue localization and expression

AR predominantly localized to nuclei of stromal and epithelial cells throughout all stages of PRCA progression (Fig. 1A). Using the tissue segmentation function of the machine learning software, we quantified AR positivity in both the epithelium and the stroma. In accordance with previously published data [10,25], the percent of AR positivity in the epithelial compartment increased in HGPIN, PRCA, and MET compared with BPT ($P<.001$ for HGPIN, $P<.0001$ for PRCA and MET; Fig. 1B). Within the stroma, AR positivity was increased in HGPIN and PRCA compared with BPT ($P<.05$ and $P<.01$, respectively; Fig. 1C). There was no difference in positivity between BPT and MET ($P>.05$)

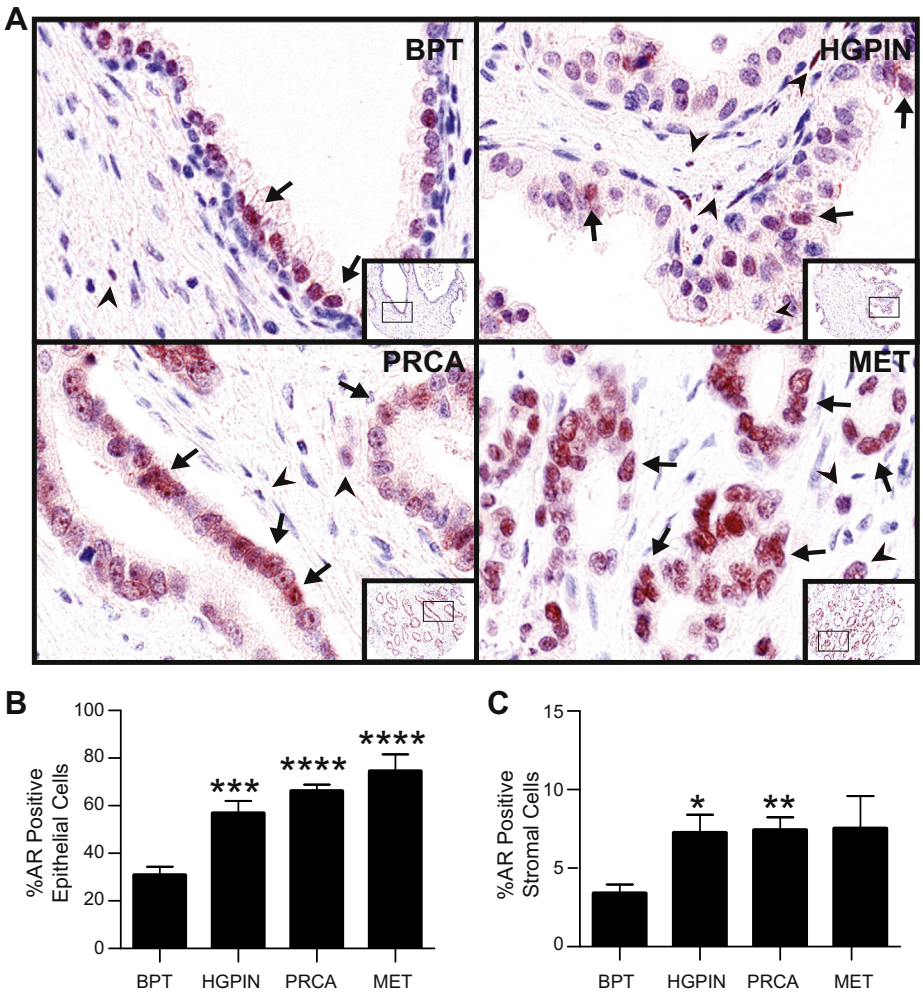


Fig. 1 AR expression in PRCA progression. A, Prostate composite image shows AR (red) in nuclei of luminal epithelial cells (arrows) and stromal cells (arrowheads). AR positivity is seen more in the epithelium than stroma, and overall, expression increased in PRCA progression.B, The percentage of AR-positive cells significantly increased in the epithelium in HGPIN, PRCA, and MET compared with BPT. C, The percentage of AR-positive cells in the stroma significantly increased in HGPIN and PRCA compared with BPT. IHC, original magnification ×100 (inset, ×20). *$P<.05$, **$P<.01$, ***$P<.001$, ****$P<.0001$ via post hoc comparison to BPT.

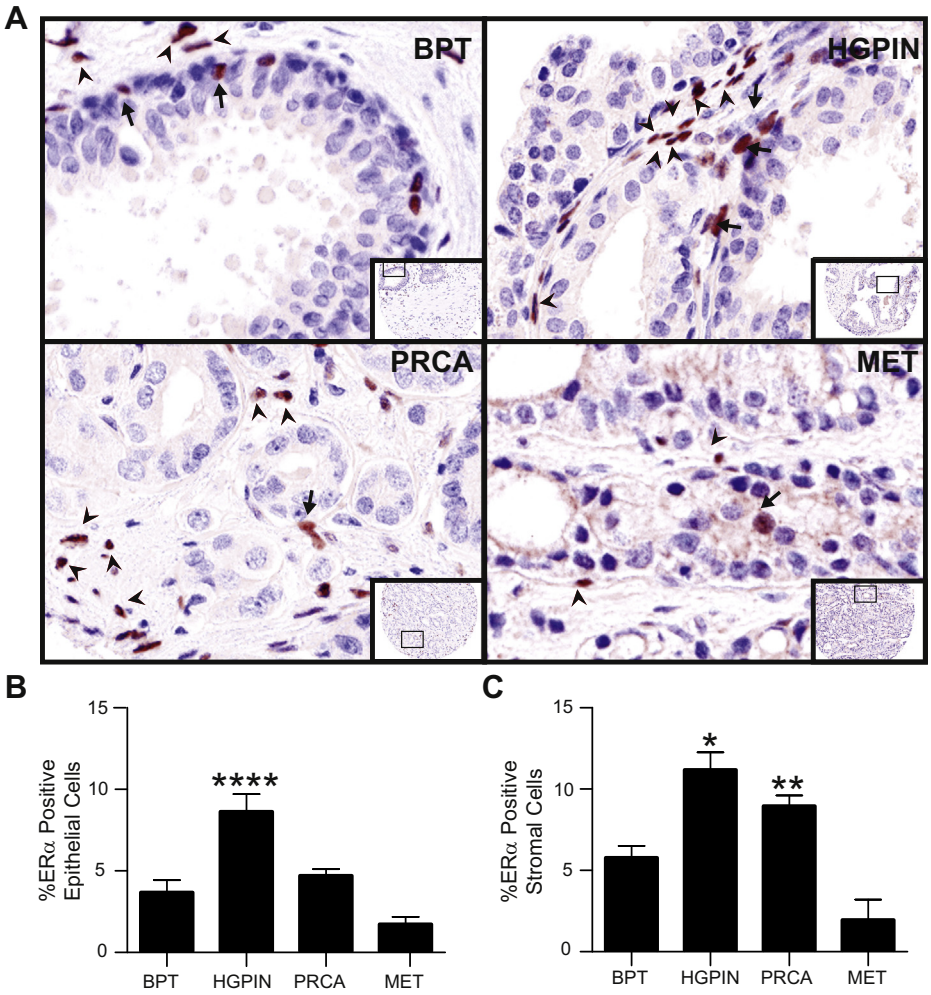


Fig. 2 ERα expression in PRCA progression. A, Prostate composite image shows ERα (brown) in nuclei of epithelial (arrows) and stromal cells (arrow heads). In normal prostate epithelium, ERα was localized primarily in basal epithelial cells, whereas epithelial ERα expression in HGPIN and PRCA showed a marked increase. B, The overall percentage of ERα positive cells in the epithelium is significantly increased in HGPIN compared with BPT. C, The percentage of ERα positive stromal cells was increased in HGPIN and PRCA compared with BPT. IHC, original magnification ×100 (inset, ×20). *P<.05, **P<.01, ****P<.0001 via post hoc comparison to BPT.

(Fig. 1C). In addition to expression trends through progression, we also noted both intersample and intrasample heterogeneity of AR expression, which was categorized as high, medium, or low AR expression (Supplementary Fig. S2). Taken together, these data suggest that (1) AR is expressed in all stages of PRCA progression, though at different levels, and (2) AR in both the epithelium and the stroma may contribute to HGPIN and PRCA, whereas AR specifically in the epithelium may contribute to MET.

3.3. ERα tissue localization and expression

ERα staining both supported and refuted previously published data [12,17-20]. As expected, ERα in BPT and HGPIN was predominantly nuclear and localized to basal epithelial and stromal cells (Fig. 2A). Interestingly, in PRCA and MET, whereas ERα localized primarily to the stroma, there was a low percentage (2%-5%) of positivity and in carcinoma cells. Segmenting stromal versus epithelial staining revealed changes in percent positivity across PRCA progression within each tissue compartment. In the epithelium, ERα positivity was increased in HGPIN (P<.0001) compared with BPT (Fig. 3B). There was no change in ERα positivity between BPT and PRCA or METS (P>.05; Fig. 2B). In the stroma, ERα positivity increased in HGPIN (P<.05) and PRCA (P<.01) compared with BPT (Fig. 2C). There was no change in ERα positivity between BPT and METS (P .05; Fig. 2C). Taken together, these data show that ERα is expressed in both the epithelium and stroma in PRCA progression. In addition, the highest percent positivity for ERα occurred in HGPIN for both tissue compartments, suggesting that this stage is where ERα contributes to prostatic disease.

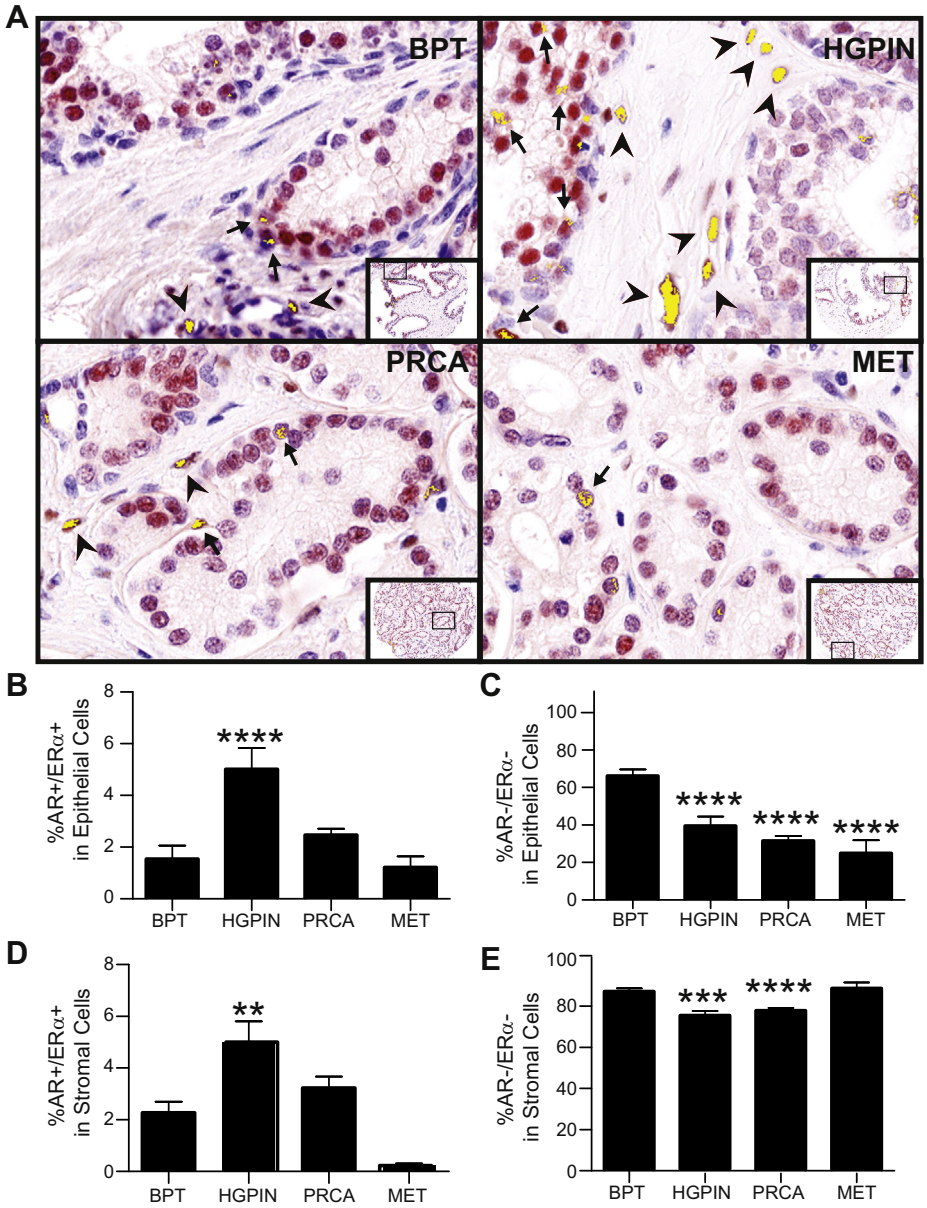
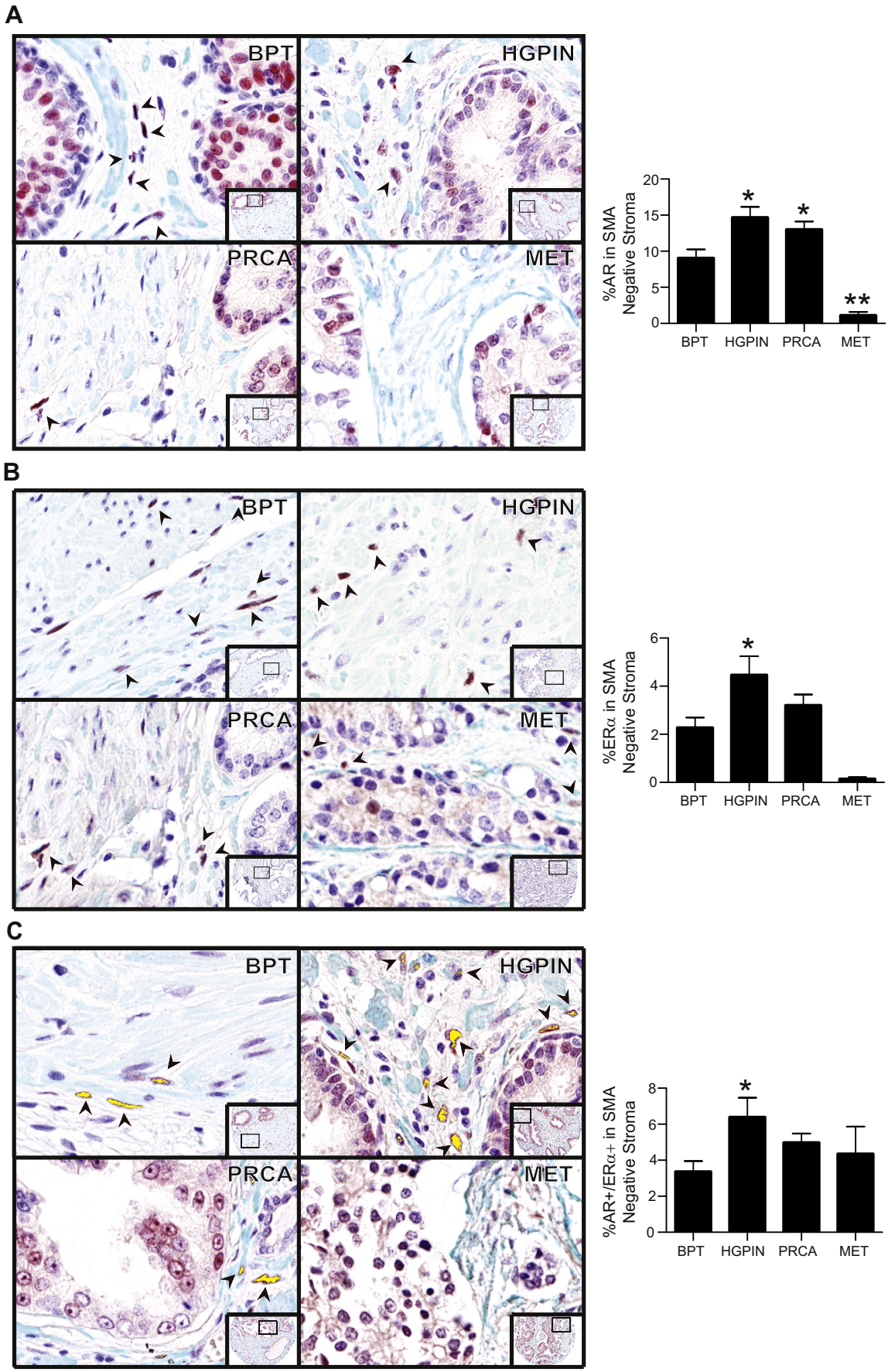


Fig. 3 Colocalization of AR and ERα in normal and pathologic prostate. A, Prostate composite image shows a small number of $AR^+/ER\alpha^+$ double-positive nuclei (yellow) seen within the epithelium (arrow) and stroma (arrowheads) in all stages of PRCA progression. B, The percent positivity of $AR^+/ER\alpha^+$ cells in the epithelium was increased in HGPIN only compared with BPT. C, Double-negative ($AR^-/ER\alpha^-$) cells in the epithelium were significantly decreased in HGPIN, PRCA and MET compared with BPT. D, In the stroma, $AR^+/ER\alpha^+$ double-positive cells were significantly higher in HGPIN compared with BPT. E, Double-negative ($AR^-/ER\alpha^-$) cells in the stroma were significantly decreased in HGPIN and PRCA compared with BPT, but unchanged in MET compared with BPT. IHC, original magnification ×100 (inset, ×20). $^{**}P$ .01, $^{***}P<.001$, $^{****}P<.0001$ via post hoc comparison to BPT.

Fig. 4 AR and ERα expression and colocalization in SMA negative stroma. A, Prostate composite image shows localization of AR (red) and SMA (turquoise) in PRCA progression. Quantification of AR in SMA-negative stroma showed a significant increase in HGPIN and PRCA compared with BPT, but a significant decrease in MET compared with BPT. B, Prostate composite image shows localization of ERα (brown) and SMA (turquoise) in PRCA progression. Quantification of AR in SMA-negative stroma showed a significant increase in HGPIN compared with BPT, and no significant change in PRCA or MET compared with BPT. C, Prostate composite image shows colocalization of cells expressing both AR and ERα (yellow) and SMA (turquoise) in PRCA progression. Quantification of $AR^+/ER\alpha^+$ cells in SMA-negative stroma showed a significant increase in HGPIN compared with BPT, and no significant change in PRCA or MET compared with BPT. IHC, original magnification ×100 (inset, ×20). $^{*}P<.05$, $^{**}P<.01$ via post hoc comparison to BPT.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3.4. Tissue coexpression and colocalization of AR and ERα

Because there may be synergistic effects between AR and ERα, we assessed the coexpression of these 2 receptors in PRCA progression (Fig. 3A). In all stages of PRCA, progression nuclear coexpression of these 2 receptors was detectable (Fig. 3A). Within the epithelium, the proportion of $AR^+/ERα^+$ increased from BPT to HGPIN ($P<.0001$, Fig. 3B) but did not change in PRCA or MET. Similarly, within the stroma, $AR^+/ERα^+$ double positivity increased from BPT to HGPIN ($P<.01$, Fig. 3D) but did not change in PRCA or MET. These data show evidence of colocalization of AR and ERα in PRCA progression, in both the epithelial and stromal compartments; however, the incidence of this coexpression is relatively low, ranging from <1% to 5%. In addition to quantification of AR/ERα coexpression by double positivity, the percentage of cells within each tissue compartment that lack both receptors can be determined. The proportion of $AR^-/ERα^-$ was lower in HGPIN, PRCA, and MET compared with BPT ($P<.0001$ for all) for the epithelial compartment (Fig. 3C). For the stromal compartment, there was a significant decrease in $AR^-/ERα^-$ cells in HGPIN and PRCA compared with BPT ($P<.001$ and $P<.0001$, respectively), but no change in MET compared with BPT ($P>.05$; Fig. 3E). Taken together, these data support the idea that as PRCA progresses, there is increased expression of steroid hormone receptors in epithelial cells as seen by a decrease in double negativity through progression. Interestingly, although the incidence of AR/ERα coexpression is relatively low overall, there is a significant increase in AR and ERα coexpression in HGPIN, suggesting that steroid receptor interaction may contribute to disease initiation.

3.5. Localization and colocalization of AR and ERα in SMA-negative stroma

Prostatic stromal tissue is made up of a variety of different cell types: fibroblasts, myofibroblasts, endothelial cells, immune cells, and others [26]. A preliminary assessment of cell type specificity for AR/ERα expression in the stroma was conducted using SMA as an initial criterion to identify differentiated smooth muscle cells and myofibroblasts. Specifically, we assessed AR/ERα expression, both separately and coexpressed, in SMA-negative stroma, which may represent cell types such as fibroblasts, immune cells, or endothelial cells. We observed an increase in the prevalence of AR-positive cells in SMA-negative stroma in HGPIN and PRCA (P .05 for both) but a decrease in AR positivity in SMA-negative stroma in MET ($P<.01$; Fig. 4A). The number of ERα-positive SMA-negative stromal cells was increased in HGPIN compared with BPT ($P<.05$), but no difference was observed in PRCA or MET (Fig. 4B). The number of $AR^+/ERα^+$, SMA-negative cells was increased only in HGPIN ($P<.05$; Fig. 4C). Taken together, these data indicate that stromal AR and ERα expression in early PRCA progression may have cell type specificity that has not previously been described.

4. Discussion

The interplay between androgens and estrogens is an aging event that has been established as a crucial component of PRCA initiation and progression [9,22]; however, the literature regarding the expression and localization of AR and ERα in PRCA progression is inconsistent [11-13,18,19]. Therefore, the incidence, stage specificity, and cell type specificity of steroid hormone receptor interactions remain unresolved. Using multiplexed IHC followed by multispectral imaging, we showed that the independent expression of steroid hormone receptors AR and ERα changes through PRCA progression. These expression and localization findings support the conclusion that androgens are important at all stages of PRCA progression, whereas estrogens, working via ERα, likely play a role in early carcinogenesis. Interestingly, the staining pattern for AR also demonstrated the presence of AR low or negative cells throughout progression, which has previously been described as AR heterogeneity [27-29]. In this study, there was both intersample and intrasample heterogeneity of AR expression ranging from samples with very low AR expression to very high. Although prostate luminal epithelial cells are generally considered to be AR positive, we found a range of 20% to 70% of epithelial cells within a core that were considered AR negative at all stages of PRCA progression. In PRCA, an average of 32% of cells within each core was considered AR low/negative in our study. Importantly, previous reports have correlated AR heterogeneity with poor patient outcomes [27-29]. Although the potential role of AR negative cells in PRCA progression remains to be fully understood, this study provides evidence of AR heterogeneity in human PRCA specimens.

To our knowledge, no other studies have reported that AR and ERα are colocalized in the same cell and that the prevalence of these double-positive cells changes with PRCA progression. This may be due, in part, to technical challenges associated with evaluating multiple markers with IHC, especially with spatially overlapping targets. In addition, steroid receptor positivity may vary based on focal areas (ie, inflammation) and be unevenly distributed throughout the prostate. Conventional methods of analysis involve visually estimating staining intensity and evaluating hotspots. To circumvent limitations of current approaches, this study used an automated pathology analysis platform to quantify expression and localization of AR and ERα in PRCA progression using 340 patient specimens [23]. This platform allows for objective quantification of the prevalence of these hormone receptors within each tissue compartment (stroma, epithelium), both separately and together; importantly, the expression output reflects average OD of each core as a whole, not a

selected hot spot. Although machine learning–based approaches to data analysis are more objective, one limitation of this technique is that results rely on the efficacy of the applied algorithm; in this study, nuclear segmentation was determined based on hematoxylin, which identifies nuclei. Therefore, cells that lack nuclei (ie, platelets, red blood cells) were not included in the analysis [23]. In addition, the tissues stained in the current study are duplicate cores taken from a larger tissue. Because of the high degree of heterogeneity within the prostate, these cores may not represent the prostate as a whole. Finally, these samples lack associated clinical data, resulting in a dearth of clinicopathologic outcomes associated with AR/ERα coexpression. Despite the limitations to automated pathology platforms [23,24], they remain a preferred approach owing to efficiency and internal consistency.

Using this technology, we report the presence of AR/ERα double-positive cells that may represent a subpopulation of cells mediating sex steroid synergy. In addition, this study provides evidence that there are at least 2 distinct populations of hormone-responsive stromal cells (AR^+ and $ER\alpha^+$) in PRCA. Although this is an enticing area for future study, the implication of the present findings is that selective steroid receptor modulators directly targeting hormone receptors might be most effective when used for prevention of PRCA carcinogenesis. Taken together with former studies demonstrating that stromal receptors are critical in PRCA progression [9,24], therapies targeting stroma may be key to the prevention of PRCA development or progression. To evaluate AR and ERα in the stromal microenvironment, we used SMA to identify differentiated smooth muscle cells and myofibroblasts, as well as to aid in the identification of SMA-negative stroma. Although this criterion does not definitively determine cell identity, due to the morphology and lack of SMA expression in these cells, it is likely that they are fibroblasts—an abundant cell type within the prostatic stroma [30,31]. Fibroblasts secrete growth factors and inflammatory cytokines, produce extracellular matrix proteins, and can create a niche for cancer cells. In cancer, these cells are referred to as carcinoma-associated fibroblasts and play a role in evading immunosurveillance, promoting a chronic inflammatory environment, and altering the metabolism of the microenvironment toward a protumorigenic state [26,32]. The present findings indicate that the stromal microenvironment of HGPIN contains an increased amount of double-positive ($AR^+/ER\alpha^+$) cells compared with BPT, which may be an important intersection in suppression or promotion of carcinogenesis. Although this study provides preliminary evidence of cell type specificity for AR and ERα coexpression, a more definitive cell type identification is necessary to understand more completely the role of fibroblasts in this complex system.

Underscoring the importance of androgens and estrogens in PRCA, we demonstrate AR and ERα expression in carcinoma cells and the stromal microenvironment changes with different stages of PRCA. Using an automated quantitative pathology approach, we report tissue-specific expression and localization of AR, ERα, and cells expressing both receptors in PRCA progression. Our findings highlight the importance of disease stage, tissue, and cell type specificity of this colocalization, and provide a foundation for further investigation into the interaction of steroid hormone receptor in PRCA. A better understanding of hormone receptor expression through PRCA progression could increase our knowledge of events contributing to PRCA initiation or progression and potentially provide rationale for cotargeting these receptors for PRCA prevention or therapy.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2019.04.009.

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