Optimization and comprehensive characterization of a faithful tissue culture model of the benign and malignant human prostate

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Few preclinical models accurately depict normal human prostate tissue or primary prostate cancer (PCa). *In vitro* systems typically lack complex cellular interactions among structured prostatic epithelia and a stromal microenvironment, and genetic and molecular fidelity are concerns in both *in vitro* and *in vivo* models. 'Tissue slice cultures' (TSCs) provide realistic preclinical models of diverse tissues and organs, but have not been fully developed or widely utilized for prostate studies. Problems encountered include degeneration of differentiated secretory cells, basal cell hyperplasia, and poor survival of PCa. Here, we optimized, characterized, and applied a TSC model of primary human PCa and benign prostate tissue that overcomes many deficiencies of current *in vitro* models. Tissue cores from fresh prostatectomy specimens were precision-cut at 300 μm and incubated in a rotary culture apparatus. The ability of varied culture conditions to faithfully maintain benign and cancer cell and tissue structure and function over time was evaluated by immunohistological and biochemical assays. After optimization of the culture system, molecular and cellular responses to androgen ablation and to piperlongumine (PL), purported to specifically reduce androgen signaling in PCa, were investigated. Optimized culture conditions successfully maintained the structural and functional fidelity of both benign and PCa TSCs for 5 days. TSCs exhibited androgen dependence, appropriately undergoing ductal degeneration, reduced proliferation, and decreased prostate-specific antigen expression upon androgen ablation. Further, TSCs revealed cancer-specific reduction of androgen receptor and increased apoptosis upon treatment with PL, validating data from cell lines. We demonstrate a TSC model that authentically recapitulates the structural, cellular, and genetic characteristics of the benign and malignant human prostate, androgen dependence of the native tissue, and cancer-specific response to a potentially new therapeutic for PCa. The work described herein provides a basis for advancing the experimental utility of the TSC model.

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Prostate cancer (PCa) cell lines and animal models have been extremely valuable in PCa research, but inherent drawbacks hinder their clinical relevance.[1,2] Cell lines, often artificially immortalized, acquire mutations in culture and lack the cellular interactions with a prostate microenvironment that are critical for prostate function and tumorigenesis.[3] Animal models may not physiologically or genetically represent true human prostate pathology.[1,2,4] There are currently few models of primary PCa, which historically has been difficult to maintain in culture or *in vivo*.[5]

To address these limitations, we and others have developed an *ex vivo* 'tissue slice culture' (TSC) model of the benign and malignant human prostate. TSC purports to be an authentic model because it preserves native tissue architecture and functional differentiation, maintaining cellular heterogeneity and complex cell–cell interactions within the intact microenvironment. TSC has been a useful practice with other organs,[6–8] and the advantages of TSC compared with monolayer cell culture are illustrated in many studies.[9–11] Of note, the intact tumor microenvironment allows stromal–epithelial interactions that are critical for realistic studies of tumor metabolism.[12,13] With collaborators, we found that TSCs exhibit steady-state glycolytic and phospholipid metabolism that mirrors that of human PCa *in vivo* but is not exhibited in PCa cell lines.[14] Such deviations from human physiology often result in inaccurate preclinical

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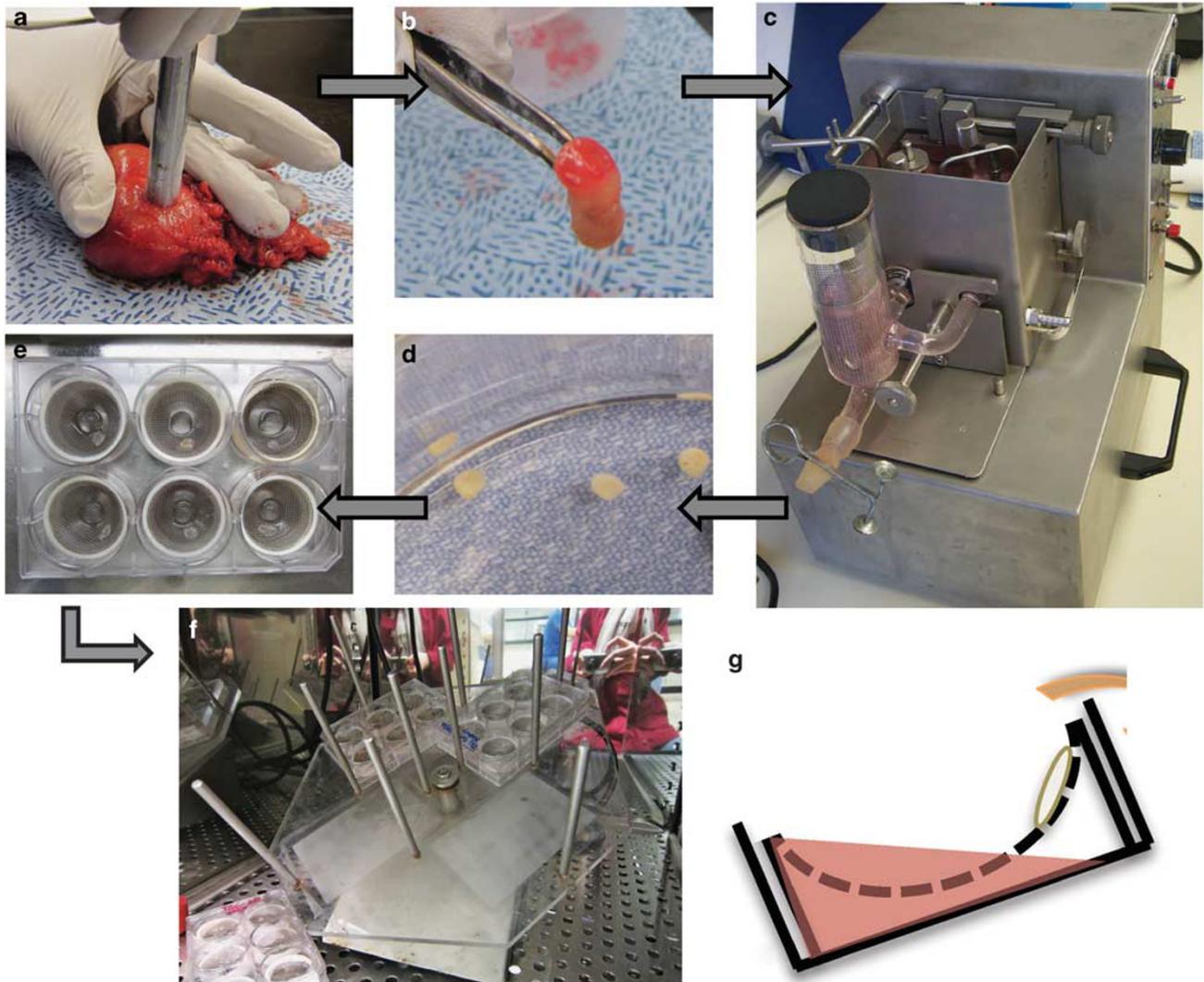E-mail: dpeehl@stanford.edu

Figure 1 Schematic for tissue slice acquisition and culture. Cores 8-mm in diameter were taken from radical prostatectomy specimens (**a**, **b**) and placed in the Krumdieck tissue slicer (**c**) from which slices emerged in a sequential manner (**d**). Slices were placed on titanium mesh inserts in six-well plates with 2.5 ml culture medium (**e**). Plates were mounted on an angled rotating platform in a tissue culture incubator (**f**). The angled rotation caused intermittent submersion of the tissue slices, facilitating diffusion of gas and nutrients from all sides of the slices (**g**).

assessment of drug responses in cell lines or animal models, leading to wasted efforts on clinical trials with drugs that are unlikely to work. TSCs, however, show promise in better-predicting drug responses in humans.[13,15]

Ex vivo culture of the human prostate has been problematic, with benign tissues often exhibiting degradation of luminal epithelial cells and hyperproliferation of basal cells.[16–18] Maintenance of PCa tissue *in vitro* has presented even more challenges than benign tissue.[16,19] Relatively recent technological innovations, particularly the practice of precision-cut slicing,[6] have led to the current form of the prostate TSC model in which 250–500 μm thick slices of tissue, 5–8 mm in diameter, are cultured under defined conditions.[18–23] Precision-cutting reduces sources of error due to variations in slice thickness and damage to cut surfaces, which both contribute to uneven gas and nutrient

exchange throughout tissue slices. It enhances reproducibility when working with heterogeneous tissues such as prostate, allowing adjacent slices to be evaluated for histology and compared pairwise under different experimental conditions. In addition, benign and PCa tissues may be compared from the same specimen. Variations of prostate TSC have been reported with mixed results.[18,19] With collaborators, we were the first to report the experimental implementation of a normal prostate TSC model, identifying altered DNA damage response mechanisms by which prostatic epithelia may be predisposed to malignant transformation.[21,22,24]

Although these studies underscore the novel experimental potential of prostate TSCs, the model remains underutilized. This is in large part because of the need for further optimization and thorough characterization of the model as well as for additional feasibility studies to encourage its use. The

Table 1 Antibody information

Company	Name	Specifications	Dilution	Incubation
Santa Cruz Biotechnology,	CK18	Mouse monoclonal	1:500	4 °C overnight
Santa Cruz, CA, USA	AR	Rabbit polyclonal	1:500	4 °C overnight
	PSA	Mouse monoclonal	1:100	4 °C overnight
	SYP	Mouse monoclonal	1:100	30 min room temperature
	ERG	Rabbit polyclonal	1:100	1 h room temperature
Biocare Medical, Concord, CA, USA	Ki67	Mouse monoclonal	Pre-diluted	1 h room temperature
	CC3	Rabbit polyclonal		1 h room temperature
	CD31	Mouse monoclonal		1 h room temperature
	AMACR/p63/CK5	Mouse mono/rabbit poly		1 h room temperature
	4plus HRP Universal 2°	Anti-mouse/anti-rabbit		30 min room temperature
Abcam, Cambridge, MA, USA	p63	Mouse monoclonal	1:200	4 °C overnight
	SMA	Mouse monoclonal	1:1000	
	CD68	Mouse monoclonal	1:500	
	PSMA	Mouse monoclonal	1:200	
Cell Signaling Technology, Danvers, MA, USA	PTEN	Rabbit monoclonal	1:100	4 °C overnight

ability to culture primary PCa is a unique feature of TSC that will confer greater authenticity to preclinical studies. One of the few reports involving PCa TSC was a pharmacodynamic profiling study reporting that TSCs were reproducible and accurate models for preclinical evaluation of drug responses.[15] As evidenced in other TSC systems, extending viability in culture beyond 2–3 days allows modeling of chronic as well as acute toxicity studies, including the metabolic activity of the tissue.[25–27]

Our objectives were to systematically optimize and comprehensively characterize both benign and PCa TSCs in order to potentiate the utility of the model. Modifications of the culture system permitted maintenance of benign differentiated secretory epithelia in the absence of basal cell hyperplasia for up to 5 days. Primary PCa of Gleason grades 3, 4, and 5 also remained viable under optimized conditions. We showed that TSCs are androgen responsive and maintained the microenvironment and heterogeneity of the native tissue, qualities that support their use as an authentic preclinical model for PCa. Finally, we used TSCs to test the cancer-specific effects of the natural compound piperlongumine (PL), which was recently evaluated in PCa cell lines.[28]

MATERIALS AND METHODS
Culture of Prostate Tissue Slices
Information about donors and tissues is presented in Supplementary Table 1. Figure 1 illustrates the acquisition, generation, and culture of prostate TSCs. Radical prostatectomy specimens were obtained immediately post surgery and 8-mm diameter cores of putative benign and PCa regions were taken from the peripheral zones according to needle

biopsy maps and gross analysis (Figure 1a and b). The cores were aseptically precision-cut to 300-μm thickness in the Krumdieck Tissue Slicer (Alabama Research and Development, Mundford, AL, USA),[8,29] from which they emerged in a sequential manner (Figure 1c and d). Alternating slices were formalin-fixed for paraffin-embedding or frozen and sectioned for histological verification by hematoxylin and eosin (H&E) staining (Supplementary Figure 1). Adjacent slices were transferred with sterile forceps onto titanium mesh inserts in six-well plates with 2.5 ml of culture medium and one to five tissue slices per well (Figure 1e). The plates were incubated at 37 °C with 95% air/5% CO_2 on a rotating platform set at a 30° angle (Alabama Research and Development, Figure 1f). Intermittent submersion in the medium caused by the angled rotation facilitates nutrient and gas diffusion throughout the 300-μm slices (Figure 1g), which is critical for maintaining cell viability over time. M199, keratinocyte serum-free medium (KSFM), and antibiotic/antimycotic were from Gibco Life Technologies (Grand Island, NY, USA) and were prepared as in Blauer et al.[19] Complete PFMR-4A was prepared as previously reported.[30] Dihydrotestosterone (DHT; Steraloids, Newport, RI, USA) and R1881 (Perkin Elmer, Waltham, MA, USA) stocks were prepared in ethanol (Sigma-Aldrich, St Louis, MO, USA). Piperlongumine (Indofine Chemical Company, Hillsborough, NJ, USA) was prepared in dimethyl sulfoxide (Fisher Scientific, Hampton, NH, USA).

Tissue Histology and Immunohistochemistry
Tissue slices were fixed in 10% buffered formalin overnight at room temperature. The slices were then either embedded in

OCT compound and frozen at $-80\,°C$ or dehydrated and embedded in paraffin according to standard practices. Serial 5-μm slices were cut for histological and immunohistochemical procedures. Formalin-fixed, paraffin-embedded sections were deparaffinized, rehydrated, and prepared for immunohistochemistry (IHC) as described previously.[31] The 5-μm sections were incubated with primary and secondary antibodies listed in Table 1 and labeled with alkaline phosphatase (Biocare Medical, Concord, CA, USA), Warp Red Chromogen (Biocare Medical), and/or 3,3-diaminobenzidine (Dako, Carpinteria, CA, USA) according to specifications and counterstained with hematoxylin. Immunohistochemical quantification was performed by blindly counting total cells and IHC-positive cells from three to six random $×40$ fields from each of three tissue slices per experimental condition. The percent IHC-positive cells per field was calculated and presented as the mean ± s.d. Statistical significance was determined by paired Student's t-tests.

Lactate Dehydrogenase and MTS Assays

Conditioned media were collected, spun down, and stored at $-80\,°C$. The Cytotoxicity Detection Kit Plus (lactate dehydrogenase; LDH) (Roche, Indianapolis, IN, USA) was used to measure LDH activity in conditioned media as directed. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to measure tissue slice viability. Slices were incubated in culture medium containing the MTS reagent for 2.5 h at $37\,°C$ and absorbances were measured as directed. LDH and MTS activity readouts were normalized to the weights in mg of the corresponding tissue slices and presented as means ± s.d. ($n = 3$). Statistical significance was determined by paired Student's t-tests.

RESULTS
Optimization of TSC Conditions

The lab previously developed a serum-free medium, 'Complete PFMR-4 A,' for primary culture of human prostatic cells.[30] This medium, with the addition of 10 nM of the synthetic androgen R1881, kept benign prostate TSCs viable for ~2 days before luminal degeneration occurred. We used these conditions as a starting point to further optimize prostate TSC to extend viability and maintain authentic prostate structure and function in vitro for up to 5 days.

Complete PFMR-4A medium with 10 nM R1881 was compared with a medium composed of KSFM/M199 with 1 nM DHT; this medium was previously reported to maintain normal prostate TSCs up to 7 days.[19] The media were changed every other day and TSCs from multiple prostate specimens were evaluated (Supplementary Tables 1 and 2). In our hands, the KSFM/M199-based medium caused basal cell hyperplasia and occasional luminal degeneration after 5 days of culture, whereas TSCs in Complete PFMR-4A with 10 nM R1881 exhibited luminal degeneration as previously observed

as well as occasional basal cell hyperplasia (Figure 2a and Supplementary Tables 1 and 2). IHC for markers of basal cells (p63) and actively proliferating cells (Ki67) confirmed basal cell hyperplasia in the tissues cultured in the KSFM/M199-based medium (Figure 2a). Representative negative controls for IHC are included in Supplementary Figure 2. Antioxidants N-acetyl cysteine and L-arginine have each been reported to aid survival in organotypic and cell cultures.[32–34] Each was added to the Complete PFMR-4A medium and tissue slices were evaluated histologically over time, but neither compound prevented luminal epithelial degeneration (data not shown).

Increasing the concentration of R1881 in Complete PFMR-4A revealed that 50 nM prevented luminal epithelial degeneration up to 5 days in culture (Figure 2a, Supplementary Figure 3; Supplementary Tables 1 and 2). A single layer of p63-positive basal cells topped by secretory luminal cells, with one to two Ki67-positive cells per gland, accurately reflected native ('day 0') tissue histology and phenotype (Figure 2a). Although we have not yet extensively characterized TSCs beyond 5 days, preliminary studies indicate that basal cell hyperproliferation and overall degeneration of both benign and PCa tissues become apparent after 1 week.

It has long been understood that tissue viability is enhanced when the culture medium is periodically refreshed to maintain sufficient levels of supplements and to remove metabolic waste products that can be damaging when present in the cellular environment.[35,36] This is particularly important for metabolic studies, and perfusion culture systems in bioreactors have been shown to enhance survival and realistically maintain cellular metabolism.[14,36,37] For our purposes, changing the culture medium every day appeared to enhance prostate TSC survival over changing the culture medium every other day. There was no perceived benefit to changing the medium more than once per day (data not shown).

TSC of Primary PCa of Different Gleason Grades

After extending the maintenance of benign prostate TSCs with the optimized PFMR-4A-based medium, we tested the efficacy of these conditions with PCa TSC of different Gleason grades. Using medium supplemented with 50 nM R1881 and changing media daily successfully maintained the native histology of Gleason grade 3 (low grade), 4, and 5 (high grade) PCa tissue slices up to 5 days in culture (Figure 2b; Supplementary Table 1). We used a triple IHC stain to verify the cancerous regions: the presence of cytoplasmic α-methylacyl coenzyme A racemase (AMACR) staining and coincident absence of both nuclear p63 and cytoplasmic cytokeratin 5 (CK5) staining indicate malignancy, whereas the presence of nuclear p63 and cytoplasmic CK5 staining in basal cells in the absence of cytoplasmic AMACR indicate normal tissue.[38] Benign glands can be interspersed in regions of cancer, as indicated by the arrows in Figure 2b.
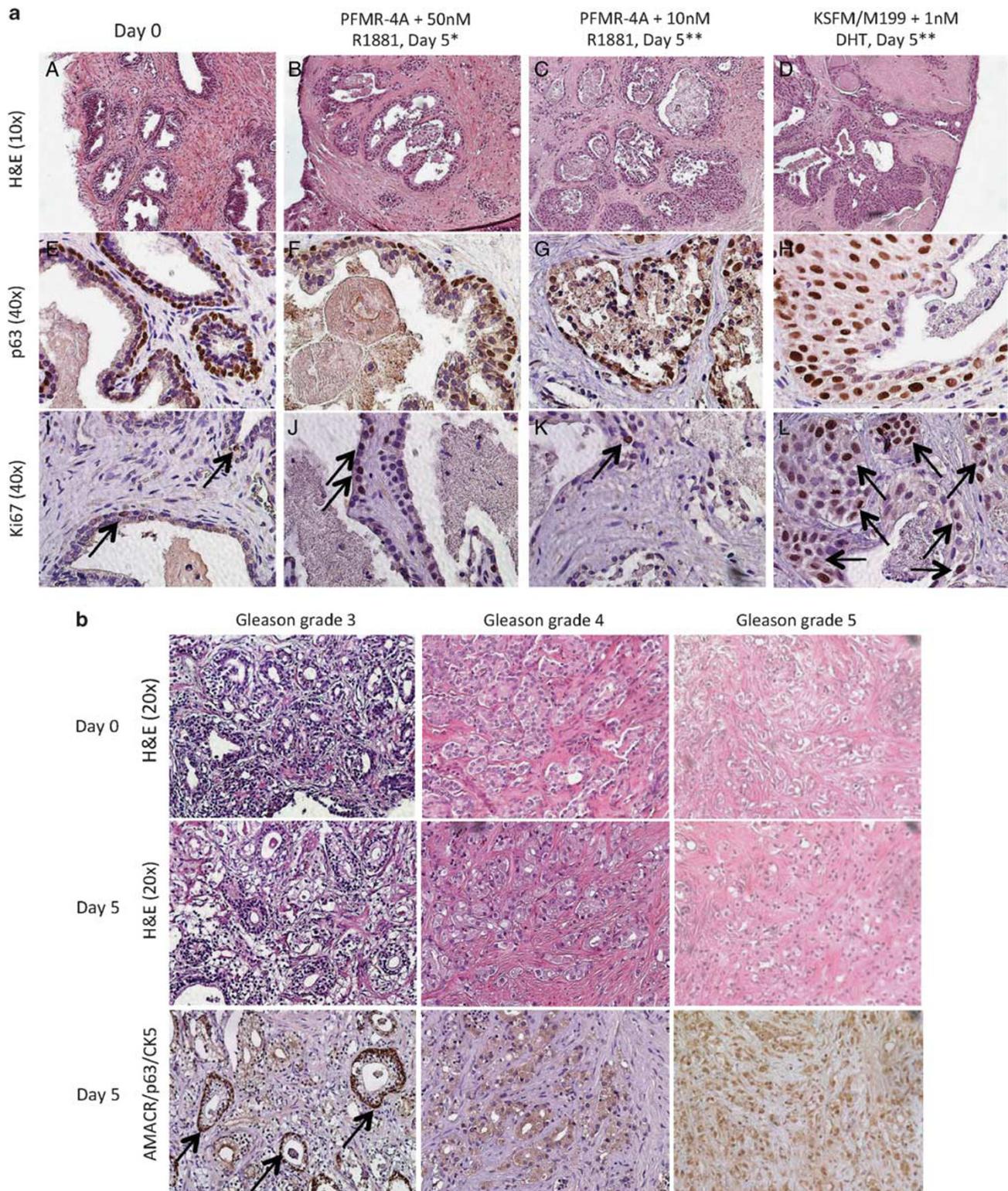
Figure 2 Histology of benign and prostate cancer (PCa) tissue slice cultures (TSCs). (**a**) Hematoxylin and eosin (H&E; **a–d**) and immunohistochemistry (IHC) for p63 (**e–h**) and Ki67 (**i–l**, arrows indicate positive nuclei) showed benign TSCs undergoing luminal cell degeneration and/or basal cell hyperproliferation compared with the native tissue ('day 0') when cultured in PFMR-4A with 10 nM R1881 (**c**, **g**, **k**) or keratinocyte serum-free medium (KSFM)/M199 with 1 nM dihydrotestosterone (DHT; **d**, **h**, **l**), but not in PFMR-4A with 50 nM R1881 (**b**, **f**, **j**) for 5 days. *Medium changed every 24 h. **Medium changed every 48 h. (**b**) Gleason grades 3, 4, and 5 PCa TSCs cultured in PFMR-4A medium with 50 nM R1881 for 5 days exhibited histologic fidelity to the native 'day 0' tissue as evidenced by H&E staining. α-Methylacyl coenzyme A racemase (AMACR)/p63/cytokeratin 5 (CK5) staining revealed regions of cancer and some interspersed benign glands (arrows). Representative images were from patient specimens number 12 (**a**), 18, 27, and 30 (**b**, Supplementary Table S1).

Optimized Culture Conditions Maintain Viability of Benign and PCa TSCs

Quantitative assays were performed to evaluate overall cell viability and cytotoxicity in TSCs in different culture conditions. MTS assays, which are biochemical measures of viability, revealed a significant improvement in the survival of benign TSCs under the optimized culture condition at day 5 compared with the other conditions tested (Figure 3a). LDH assays, which measure cytotoxicity, showed decreased cytotoxicity under optimized conditions (Figure 3b). PCa TSC viability and cytotoxicity levels were on par with those of the benign prostate TSCs in the optimized medium (Figure 3a and b). MTS and LDH assay results corresponded to the qualitative histological assessments represented in Figure 2. Further, cleaved caspase 3 (CC3), a marker of apoptosis, was not detected in TSCs cultured for 5 days in the optimized culture system (Figure 3c and d). Ki67, a marker for proliferating cells, was present in ∼2% of benign epithelial cells and ∼7% of cancer cells in both native tissue and day 5 TSCs (Figure 3d). The percentages of CC3- and Ki67-positive nuclei were quantified from three random ×40 fields from each of three tissue slices per condition (average of 97 nuclei per field), and there were no statistically significant differences between days 0 and 5 (Figure 3d). Importantly, prostate-specific antigen (PSA) continued to be expressed in the benign luminal epithelial cells and cancer cells in TSCs at 5 days, suggesting sustained prostatic function *in vitro* (Figure 3c). Together, these histological, biochemical, and immunohistochemical assays confirm the functional viability of both benign and PCa TSCs up to 5 days in culture.

Benign and PCa TSCs Maintain the Cellular Complexity of the Native Tissue

One of the major advantages of the TSC model over current *in vitro* models is its potential to preserve the structural and cellular diversity of the human prostate. Cellular and molecular heterogeneity and dependence on endocrine and paracrine signaling are important elements of human PCa that are lacking in current *in vitro* models. Cross talk between the epithelia and the stromal microenvironment, which includes fibroblasts, vasculature, nerves, and cells of the immune system, is critical to functions of the normal prostate as well as to cancer progression.[3,39,40] Benign and cancer tissues were obtained from the same specimen and IHC was performed for lineage-specific markers of the various cell types within the human prostate at day 0 (immediately post surgery) and in adjacent TSCs after 5 days (Figure 4). The basal epithelial cell marker (p63) was present in a single layer in benign glands but was absent from malignant glands as expected, whereas cytokeratin 18 (CK18) was present in both benign luminal epithelia and cancer, as appropriate. Smooth muscle α-actin (SMA) was present throughout the stroma as expected in both benign and cancer tissues. CD31 staining of endothelial cells showed evidence of vasculature in the stroma, more so in regions of cancer as has been previously

observed.[41] CD68 is a cell surface glycoprotein expressed by macrophages and monocytes, and its increased presence in cancer compared with benign tissue reflects an inflammatory phenotype common to PCa.[40] Finally, a marker for neuroendocrine cells, synaptophysin, was expressed in distinct cells within the basal cell layer in the benign tissue and in sporadic cancer cells as expected (Figure 4).[42] Overall, these results demonstrate that benign and cancer tissue slices cultured for 5 days in our system retain much of the cellular complexity of native tissues, validating the fidelity of the TSC model of the human prostate.

Benign and PCa TSCs Retain Biomarker Expression Patterns of Native Tissue

After confirming that prostate TSCs maintained the cellular composition of the native tissue, we next investigated maintenance of expression of biomarkers that are differentially expressed in PCa by IHC in tissue slices cultured for 5 days (Figure 5). AMACR/p63/CK5 triple staining, used to confirm the presence of PCa in clinical specimens, distinguished regions of benign and malignant tissue in TSCs. The *TMPRSS2–ERG* gene fusion, occurring in ∼40% of prostate tumors, leads to androgen-driven upregulation of the ERG transcription factor.[43] Using ERG expression as a surrogate marker for the TMPRSS2–ERG fusion,[44] we identified PCa specimens that expressed ERG but observed that ERG expression was reduced in the TSCs after 5 days in culture. Although it is possible that androgen signaling is less robust in TSCs, TMPRSS2 expression was well-maintained at day 5. Androgen receptor (AR) expression was detected in both benign and PCa-native tissues and in TSCs after 5 days in culture. PTEN was identified in both benign and PCa specimens but to a somewhat lesser extent in PCa, which may be due to haploinsufficiency.[45,46] Both benign and PCa TSCs maintained the PTEN expression of the native tissues after 5 days in culture. Prostate-specific membrane antigen (PSMA) was detected in benign and malignant glands as expected in both native tissue and in TSCs after 5 days.[47] In both native tissue and the day-5 TSCs, E-cadherin was expressed at the cell membrane of benign epithelial cells but exhibited more diffuse cytoplasmic staining in the PCa cells as expected.[48] Overall, the fidelity of protein expression patterns in TSCs to the native tissues together with their cellular and structural fidelities show that the TSC model uniquely recapitulates prostate biology on structural, cellular, and molecular levels.

Benign and PCa TSCs Exhibit Androgen Dependence

Benign human prostate and PCa are both highly dependent on androgen signaling, and we showed in Figure 5 that both benign and PCa TSCs express AR. In order to further evaluate the authenticity of the TSC model, the response of TSCs to androgen ablation was assessed. Benign and PCa TSCs were cultured in Complete PFMR-4A medium in the presence (50 nM R1881) or absence (0.05% ethanol control)
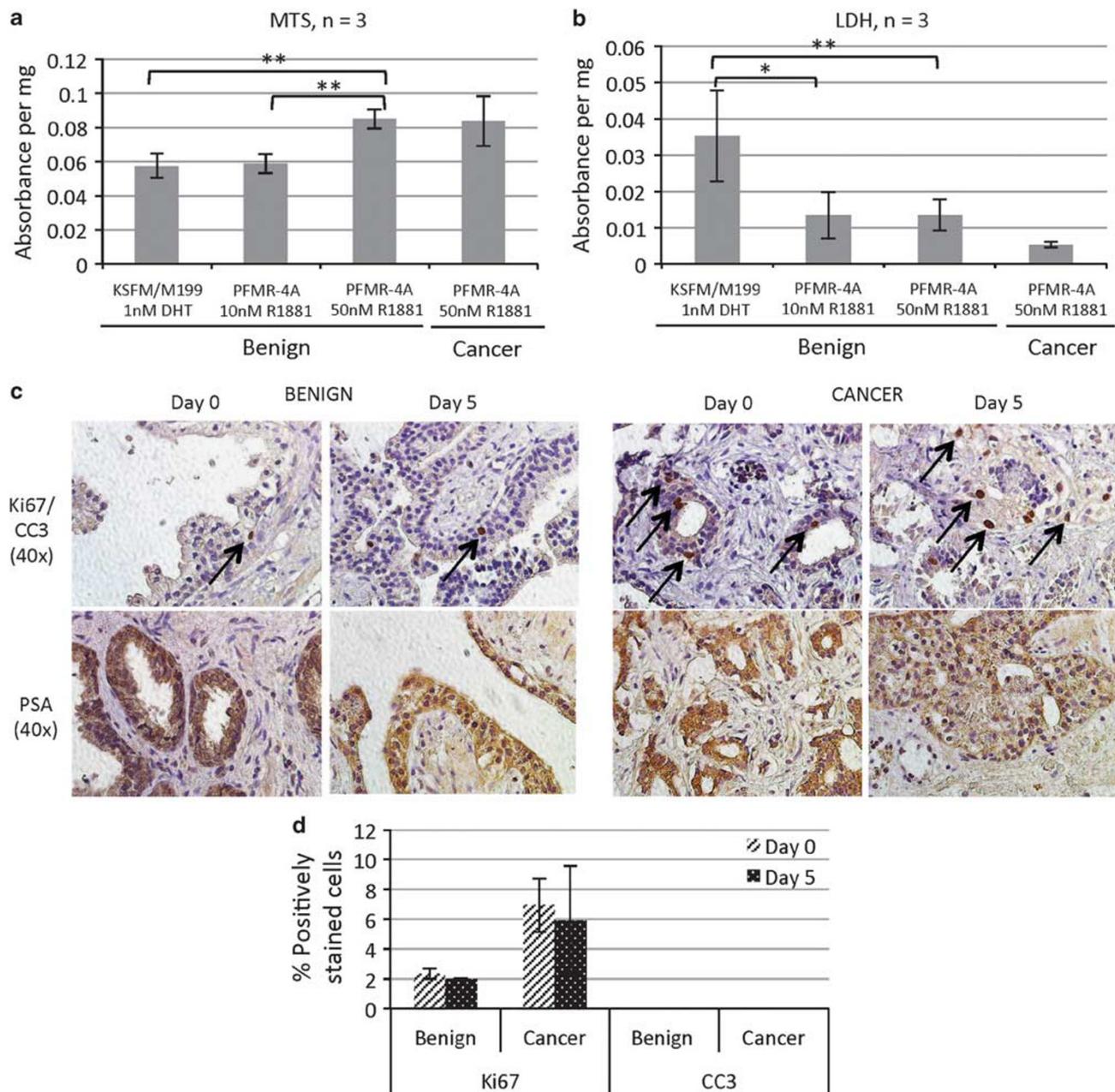
Figure 3 Tissue slice culture (TSC) viability and cellular activity. Benign TSCs cultured in PFMR-4A with 50 nM R1881 for 5 days exhibited increased viability by MTS assay (**a**) and reduced cytotoxicity by lactate dehydrogenase (LDH) assay (**b**) compared with those cultured under different conditions. Prostate cancer (PCa) TSCs exhibited levels of viability (**a**) and cytotoxicity (**b**) similar to those of benign TSCs. *$P < 0.01$, **$P < 0.001$. (**c**) Benign and PCa TSCs cultured for 5 days in PFMR-4 A with 50 nM R1881 maintained the same proliferative and apoptotic profiles as the native tissue ('day 0') as evidenced by the presence of Ki67-positive cells (arrows) and the absence of cleaved caspase 3 (CC3) staining, respectively (top row, quantified in **d**). They also continued to express prostate-specific antigen (PSA; bottom row). (**d**) The percentages of CC3- and Ki67-positive nuclei were quantified from three random × 40 fields from each of three tissue slices per condition (average of 97 nuclei per field). Experiments were performed on patient specimens number 19, 20, 21 (**a**, **b**), 14, and 18 (**c**).

of androgen. Upon histological evaluation after 5 days in culture, there was evidence of ductal degeneration in regions of both benign and cancer TSCs in the absence of androgen (Figure 6b, arrows, Supplementary Tables 1 and 2). An MTS assay showed a statistically significant reduction of viability in benign TSCs but not cancer after 5 days in the absence of androgen (Figure 6a). However, there is likely a hetero-

geneous response to androgen ablation among the cell types in both benign and cancer TSCs, as the histology suggested, which may not be revealed by the MTS assay.

We used IHC to further investigate the effects of androgen ablation on TSCs. After 5 days, evidence of proliferating cells (Ki67-positive, black arrows) decreased and evidence of apoptotic cells (CC3-positive, red arrows) increased in both
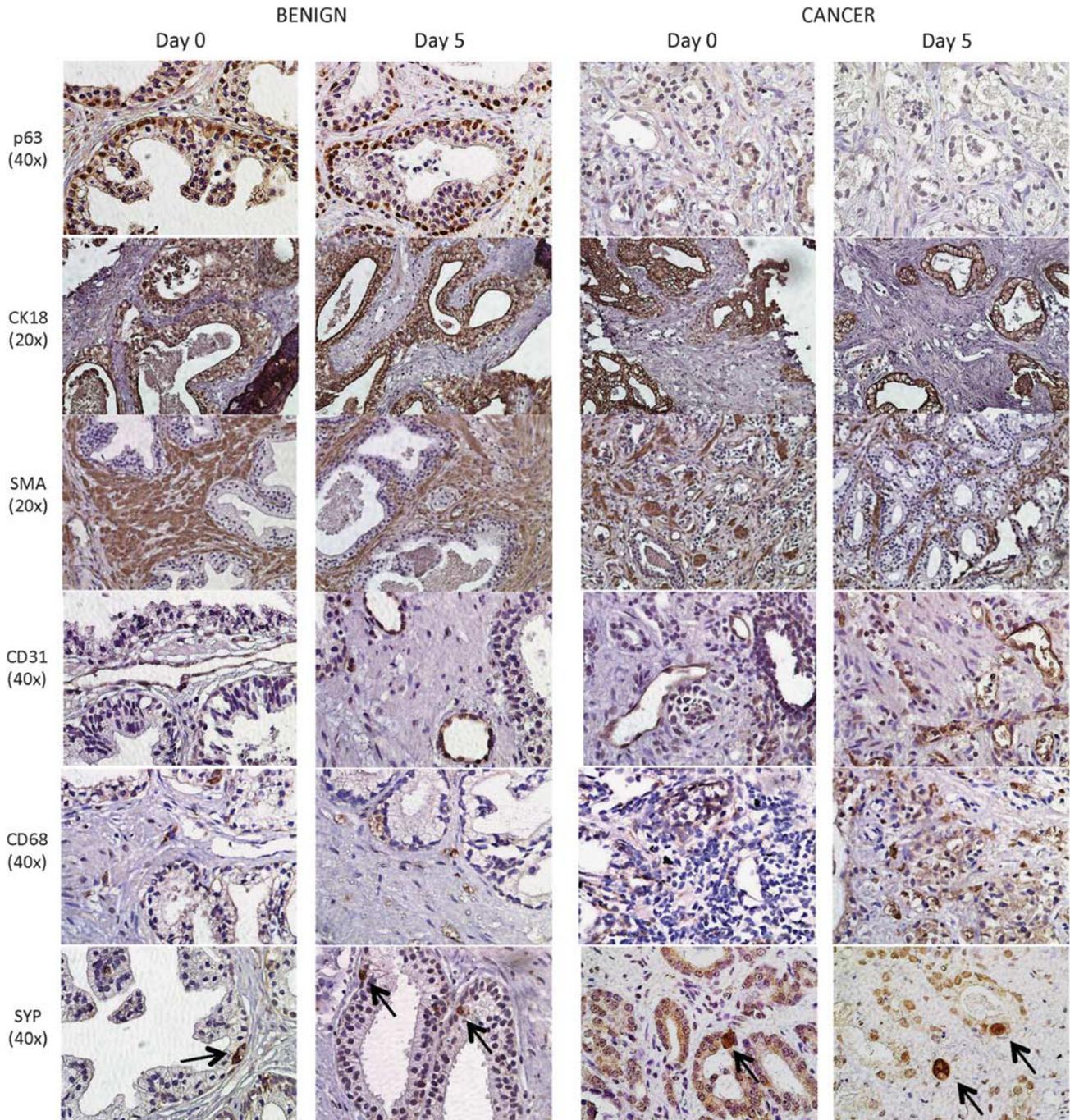
Figure 4 Cellular compositions of benign and prostate cancer (PCa) tissue slice cultures (TSCs). Cellular markers of basal epithelial cells (p63), luminal epithelial cells and cancer cells (cytokeratin 18; CK18), stromal cells (smooth muscle α-actin; SMA), endothelial cells (CD31), inflammatory cells (CD68), and neuroendocrine cells (SYP, arrows) were appropriately expressed in benign and PCa TSCs after 5 days in culture. The expression patterns recapitulated those in the native 'day 0' tissues. Representative images were from patient specimens number 16, 18, and 27.

benign and cancer TSCs in the absence of androgen (Figure 6c). Quantification of the percent positively stained nuclei from six random × 40 fields (average of 90 nuclei per field) from each of three tissue slices per condition confirmed statistically significant increases in apoptotic cells in benign and PCa TSCs in the absence of androgen (Figure 6d). There was a statistically significant reduction in Ki67-positive cells

in PCa but not benign TSCs in the absence of androgen (Figure 6d). Further, intensity of PSA, an androgen-regulated gene, diminished in both benign and cancer TSCs as expected (Figure 6c). Together, these results show the androgen dependency of both benign prostate and primary PCa TSCs, further supporting the biological relevance of the TSC model to the human prostate.

TSCs Validate a Cancer-Specific Response to Piperlongumine

Investigations to assess the feasibility of using TSC as a predictive preclinical model for human therapeutic response are ongoing across a variety of organ types.[7,49,50] Of particular value is the ability to compare responses of cancer *versus* benign cells in TSCs, as efforts to identify cancer-specific drugs with reduced side effects intensify. The naturally derived alkaloid PL was recently found to selectively kill cancer cells across a range of cell lines and in a transgenic mouse model for breast cancer.[51] Golovine *et al*[28] subsequently tested the effects of PL on PCa cell lines and reported that after only 3–6 h, PL depleted AR and reduced cell proliferation. There remains the question of whether the effects of PL on PCa cell lines will be recapitulated in the native prostatic microenvironment and whether PL will affect benign prostate cells as well as the cancer. After confirming the androgen dependence of benign and PCa TSCs, we evaluated the effects of PL in this more biologically relevant system in which we can compare benign and cancer responses.

Six-hour treatment with low-dose PL (10 μm) appeared to reduce viability and cause epithelial degeneration to a somewhat greater extent in cancer TSCs than in benign as evidenced by MTS assays and H&E staining, respectively (Figure 7a and b). We next used IHC to localize cell-specific effects of PL in the benign and cancer TSCs. AR expression persisted in benign TSCs after 6 h of PL treatment, whereas AR was significantly reduced in PL-treated PCa TSCs (Figure 7c and d). Further, while there was a slight decrease in actively proliferating (Ki67-positive) cells in the PL-treated PCa TSCs, there was a significant increase in PCa cells undergoing apoptosis (CC3-positive) compared with benign cells (Figure 7c and d). These results are consistent with and validate the findings by Golovine *et al*[28] (although they did not test for apoptosis), while additionally revealing a cancer-specific effect of PL in the prostate that is consistent with findings in other tissue types.[51] These findings lend support for further investigation into the use of PL in the clinic, and they exemplify the utility of the TSC model for validating preclinical studies previously executed only in cell lines or animal models.

DISCUSSION

Optimization of culture conditions resulted in maintenance of benign and malignant prostate TSCs for 5 days, a significant improvement over our previous ability to maintain TSCs for only ∼2 days without signs of degeneration. Although others have reportedly maintained prostate TSCs for up to a week, maintenance of structure and function was not comprehensively evaluated in those studies, and we could not reproduce those findings. We extended the longevity of TSCs by using a serum-free medium previously developed for primary culture of human prostatic epithelial cells, Complete PFMR-4A, and increasing the androgen supplement, R1881, from 10 to 50 nM. Although 50 nM is a high dose of this synthetic androgen that is often used in cell culture because of its stability compared with the natural androgen, DHT, little is known about hormone dynamics in this experimental context. Changing the medium daily also contributed to increased survival of TSCs. Although perfusion culture might prove superior,[37] frequent refreshment of medium is a feasible alternative when using standard culture apparati.

Why prostate tissues are so challenging to maintain in culture for long periods is unclear. TSCs from other species or organs can be maintained for weeks or months.[7,25,27,52] Organotypic culture models of the human prostate and PCa have taken several forms in the past, but their uses have been hampered by a short lifespan, the need for technical optimization, and lack of thorough characterization to confirm fidelity and reproducibility.

Over the 5-day period of culture in optimized medium, the TSC model maintained the authentic structure, function, and biological characteristics of both the benign prostate and PCa. Basal cell hyperplasia and luminal cell degeneration were minimized and the characteristic single layer of basal cells topped by a layer of secretory luminal cells was preserved in benign epithelial glands. Classic cell-type expression of proteins was retained, as evidenced by the localization of p63 and CK5 to the basal cells and CK18, AR, and PSA to the luminal cells. Cancer-associated markers that are specifically or differentially expressed in PCa, including AMACR, TMPRSS2, PTEN, PSMA, and E-cadherin, were appropriately expressed in PCa TSCs. Neuroendocrine differentiation in both benign prostate and PCa TSCs was typical of that observed in native tissue. SMA expression confirmed the survival of smooth muscle cells and myofibroblasts in the stroma of benign tissue and PCa, respectively. Comparable rates of proliferation and apoptosis were observed in TSCs and native tissues, as measured by labeling for Ki67 and CC3.

Recapitulation of the cellular and molecular phenotype of the native tissue is lacking in other *in vitro* models. Cellular and molecular heterogeneity, in addition to the presence of an intact microenvironment, are ultimately necessary for accurate preclinical evaluations. Our comprehensive characterization also showed for the first time that endothelial cells, macrophages, and monocytes survive in prostate TSCs. The presence of these cells may enable preclinical studies of therapeutics targeting the vascular or the immune system.

A notable outcome of optimizing culture conditions was our ability to maintain PCa of different Gleason grades in TSCs. There is a pressing need for model systems that authentically represent low-risk (Gleason grade 3) and high-risk (Gleason grades 4/5) primary human PCa, as the latter is associated with a high risk of recurrence and current neoadjuvant therapies have not significantly extended overall survival.[53] TSCs can fulfill this need, as all Gleason grades were maintained during the 5-day culture period and can be used to screen novel therapies. Further, PCa-derived TSCs often contain admixed benign glands, uniquely providing an internal control for evaluating cancer specificity and
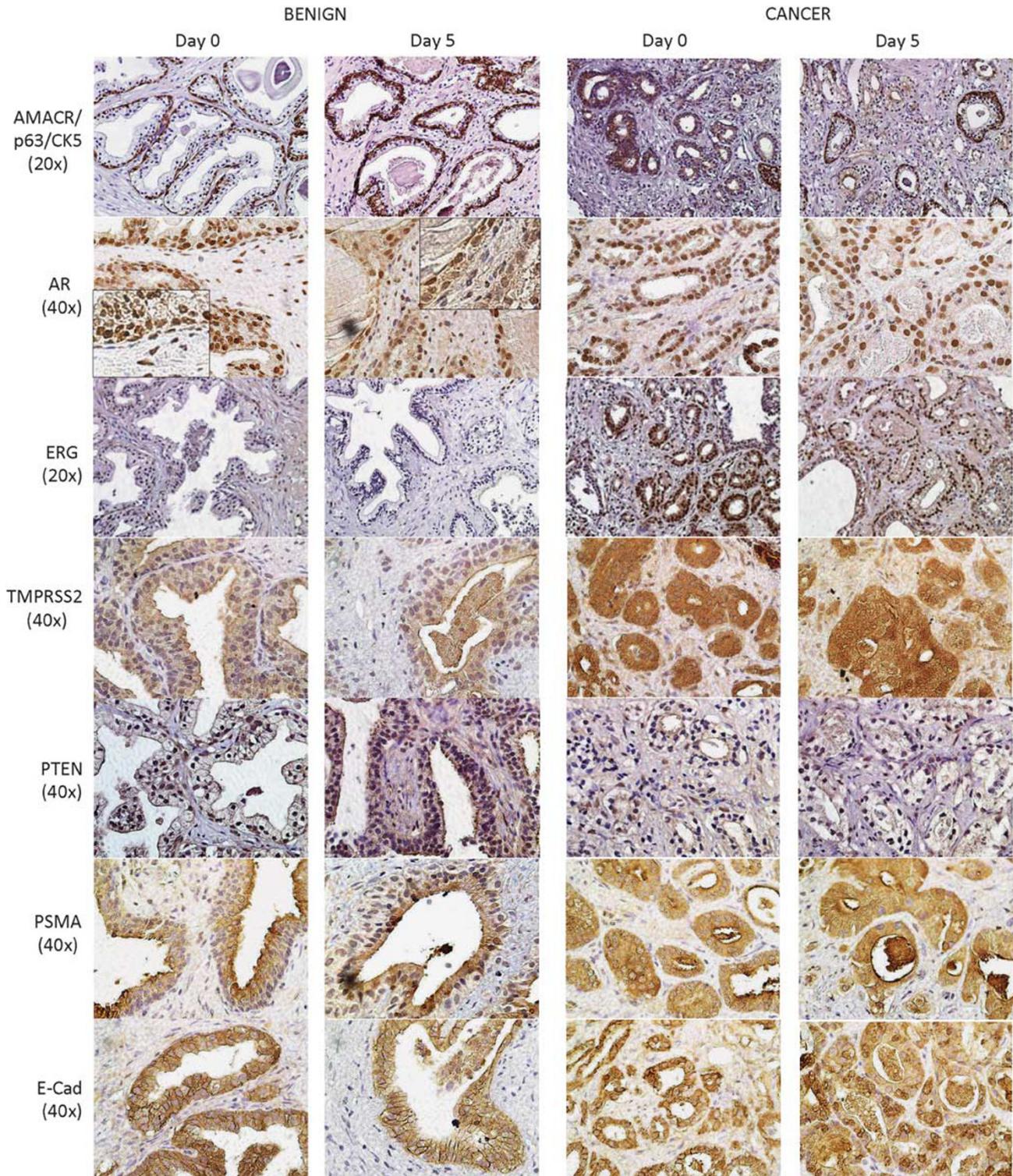
Figure 5 Biomarker expression in benign and prostate cancer (PCa) tissue slice cultures (TSCs). Immunohistochemistry (IHC) expression patterns of known biomarkers in benign and PCa TSCs cultured for 5 days mirrored those in the corresponding native 'day 0' tissues. A ×60-magnification inserts show the absence of androgen receptor (AR) staining in basal cells of benign TSCs. Representative images were from patient specimens number 18, 27, and 28.

mechanisms of experimental therapeutics. Taking advantage of this feature, we tested the activity of the natural agent PL that reportedly reduces AR expression in a cancer-specific manner.[28] We validated this finding in the TSC model, supporting further investigation into the efficacy of PL in the clinical setting.
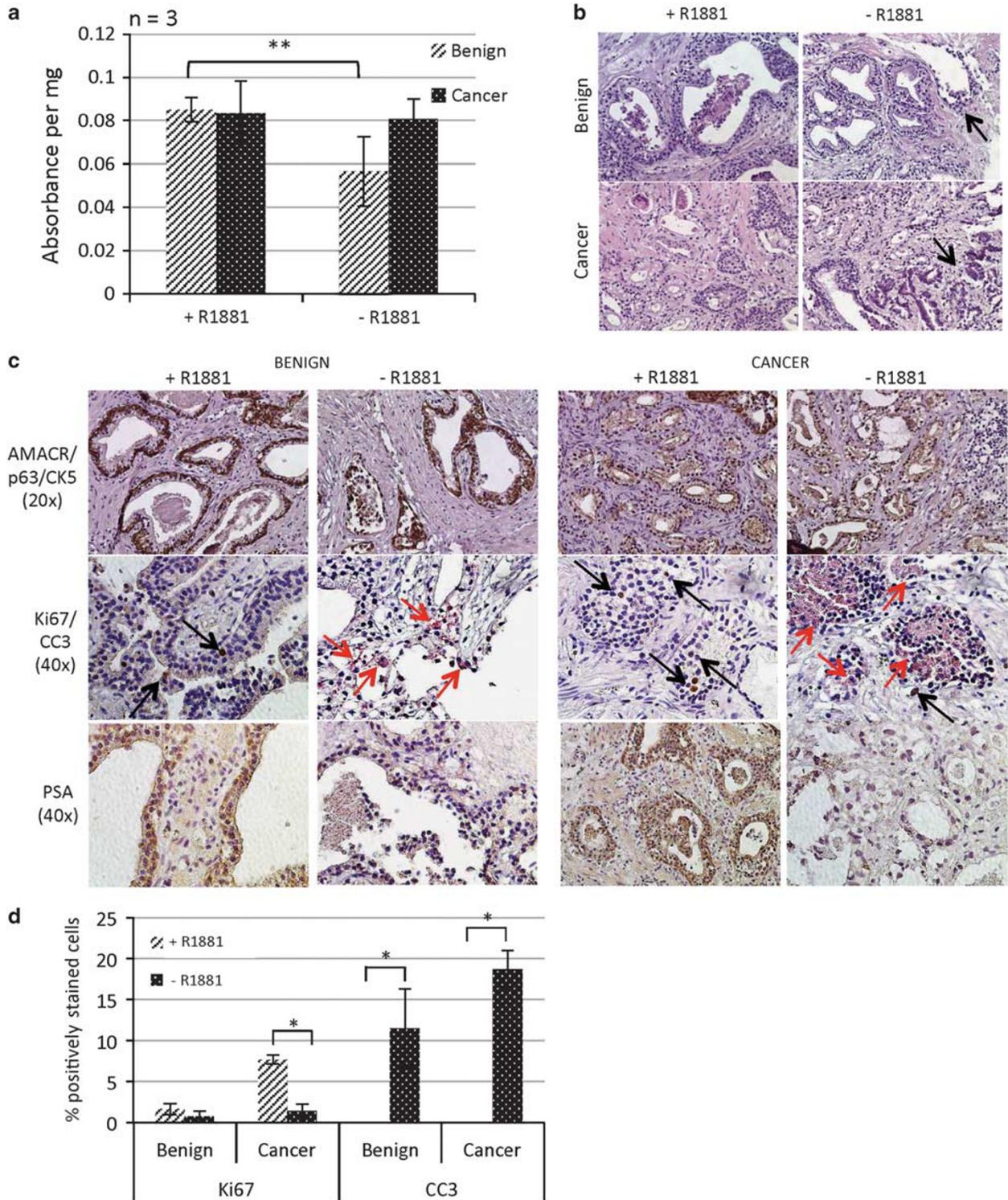
Figure 6 Responses of tissue slice cultures (TSCs) to androgen ablation. Culture of benign and prostate cancer (PCa) TSCs in the absence of androgen for 5 days resulted in heterogeneous reduction of cellular viability and glandular integrity according to MTS assays (**a**) and hematoxylin and eosin (H&E) staining (**b**, arrows point to degenerating glands), respectively. Luminal degeneration was also evident upon immunohistochemistry (IHC) evaluation of benign and PCa TSCs cultured for 5 days in the absence (ethanol control) of R1881 (**c**). Benign and PCa TSCs were verified by α-methylacyl coenzyme A racemase (AMACR)/p63/cytokeratin 5 (CK5) staining (top row). Androgen ablation reduced the number of actively proliferating cells (Ki67 positive, black arrows) and increased the number of cells undergoing apoptosis (cleaved caspase 3 (CC3) positive, red arrows) in both benign and PCa TSCs. Prostate-specific antigen (PSA) expression was reduced in both benign and PCa TSCs upon 5 days of androgen ablation (bottom row). (**d**) The percentages of CC3- and Ki67-positive nuclei were quantified from six random $\times 40$ fields from each of three tissue slices per condition (average of 90 nuclei per field). *$P < 0.05$, **$P < 0.01$. Experiments and representative images were from patient specimens number 18, 20, and 21.
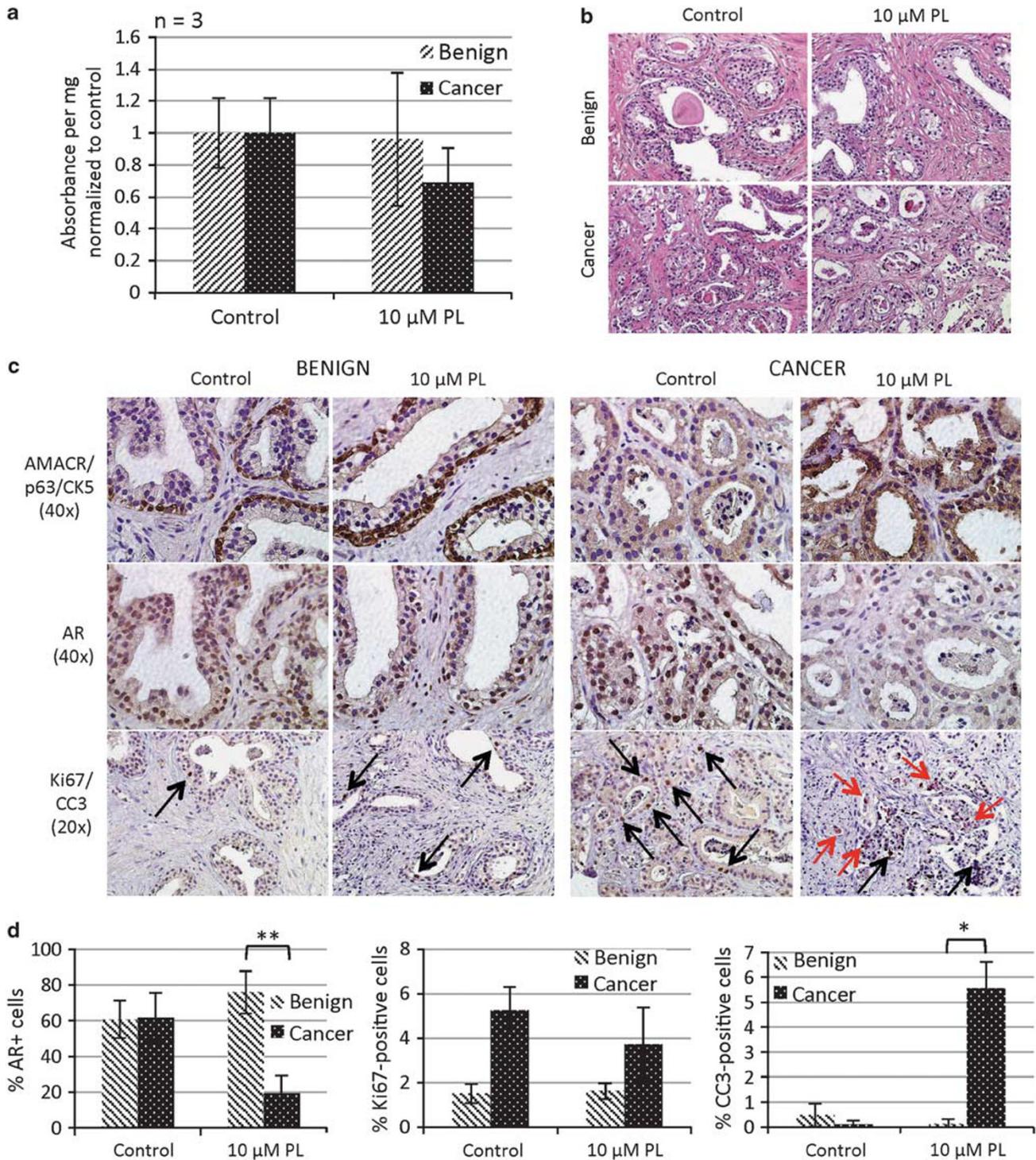
Figure 7 Responses of tissue slice cultures (TSCs) to piperlongumine (PL). Prostate cancer (PCa) but not benign TSCs treated with PL for 6 h exhibited a nonsignificant reduction of viability (MTS assay, **a**) that was mirrored by sporadic regions of luminal degeneration (hematoxylin and eosin (H&E), **b**). Further immunohistochemistry (IHC) evaluation of benign and PCa TSCs revealed a cancer-specific decrease of androgen receptor (AR) expression (middle row **c**, **d**) and increase of apoptotic cells (red arrows, bottom row **c**, **d**). PL treatment did not significantly affect proliferation among benign or PCa cells (black arrows, bottom row **c**, **d**). Total nuclei (average of 114 per field) and positively stained nuclei were blindly counted from three random $\times$ 40 fields from each of three tissue slices per treatment (**d**). $*P < 0.01$, $**P < 0.001$. Experiments and representative images were from patient specimen number 26.

The androgen dependence that we demonstrated in TSCs is an essential characteristic of an authentic model of the benign and malignant prostate, especially considering that targeting the AR signaling pathway is a major goal in PCa research and therapy. However, further investigation into the mechanism of androgen signaling in TSCs will be necessary.

Although expression of the androgen-regulated genes *TMPRSS2* and *PSA* persisted in TSCs, ERG expression (presumably driven by the androgen-responsive promoter of the *TMPRSS2–ERG* gene fusion) waned. AR signaling dynamics have not yet been investigated in this model system, and persistence of target gene expression may be due to longer half-lives instead of active AR signaling. Regardless, the presence of androgen proved to be critical for maintenance of benign and PCa TSCs.

The faithful retention of tissue structure and function in the TSC model presents an ideal opportunity to evaluate multifaceted effects of therapeutic compounds on an organ.[15] TSC may be used to identify potential biomarkers of drug response, determine drug concentrations that affect organ function or morphology, carry out gene or protein expression profiling, identify species–specific or cell-line-specific effects, and elucidate mechanisms relevant to human safety.[49] A recent study evaluated a new HSP90 inhibitor in PCa cell lines and organotypic PCa tissue cultures and reported that the tissue cultures provided information on the drug response that was not previously observed in cell lines or animal models.[50] Further studies testing the boundaries of preclinical assessment with TSCs are warranted.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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