A lack of clinically relevant experimental models of human prostate cancer hampers evaluation of potential therapeutic agents. Currently, androgen deprivation therapy is the gold standard treatment for advanced prostate cancer, but inevitably, a subpopulation of cancer cells survives and repopulates the tumor. Tumor cells that survive androgen withdrawal are critical therapeutic targets for more effective treatments, but current model systems cannot determine when they arise in disease progression and are unable to recapitulate variable patient response to treatment. A model system was developed in which stromal-supported xenografts from multiple patients with early-stage localized disease can be tested for response to castration. The histopathology of these xenografts mimicked the original tumors, and short-term host castration resulted in reduced proliferation and increased apoptosis in tumor cells. After 4 weeks of castration, residual populations of quiescent, stem-like tumor cells remained. Without subsequent treatment, these residual cells displayed regenerative potential, because testosterone readministration resulted in emergence of rapidly proliferating tumors. Therefore, this model may be useful for revealing potential cellular targets in prostate cancer, which exist before the onset of aggressive incurable disease. Specific eradication of these regenerative tumor cells that survive castration could then confer survival benefits for patients.

INTRODUCTION

In men with aggressive prostate cancer, androgen deprivation therapy elicits a rapid atrophic response and is the gold standard of therapy for advanced prostate cancer. However, tumors eventually become resistant to treatment, resulting in progression to incurable castrate-resistant prostate cancer (CRPC). There are several mechanisms by which prostate cancer cells adapt to low systemic androgens, including activating androgen receptor (AR) mutations and testosterone synthesis from adrenal androgens or de novo by the tumor cells (1–6). It is postulated that castrate-resistant stem-like cells facilitate the progression to CRPC in a stepwise process, beginning with a subset of cells that survive androgen deprivation therapy and then adapt to the presence of low systemic testosterone resulting in the emergence of CRPC (7, 8).

A central unresolved question in prostate cancer is whether the cancer cells that survive androgen withdrawal preexist in early-stage localized tumors or emerge in later stages of progressive disease. Thus, the potential cells of origin of CRPC remain unknown. Identification of such cells in localized tumors would stimulate the consideration of adjuvant treatments that target these castrate-surviving cells, especially for men who are deemed to be at high risk of relapse.

It was previously difficult to address these questions using human primary prostate cancer specimens, particularly those with early-stage, localized disease. A recent study used a metastatic xenograft cell line, BM18, to identify castrate-resistant, but hormone-sensitive, tumor cells (9). In this model, a subpopulation of the tumor cells from advanced prostate cancer persisted in the castrate milieu, exhibiting a luminal or neuroendocrine phenotype, expressed stem cell markers, and regenerated tumors upon readministration of testosterone (8). The BM18 xenograft line model represents highly aggressive/metastatic end stage of disease and does not address whether similar populations are present in all prostate tumors and at what stage of disease progression these cells arise. Most importantly, it is unknown whether equivalent cancer cells that persist during castration are present in tumors from men with earlier stages and localized disease.

Our recent report documenting the improved efficiency of xenografting primary human prostate cancer from localized tumors now provides a method to address this issue (10). The use of primary human tissues also allows individual responses of patient tissues to be tracked, unlike xenografts of cell lines that are unable to mimic patient biological diversity (11–13). Therefore, this study aims to identify and characterize these therapy-resistant cells using this efficient and reliable assay to grow primary human localized prostate cancer in vivo (10). We postulate that before the development of advanced disease, localized prostate cancer specimens may harbor tumor cells that can survive castration. This hypothesis was tested using xenografted specimens from 12 men with localized disease, which were subjected to short- and long-term castration as well as androgen restoration.

RESULTS

Xenografted prostate cancer specimens reflect pathological characteristics of patient tumors

Tumors from men with localized prostate cancer are difficult to engraft because the take rates are notoriously low (11). However, we have
recently developed a protocol in which co-grafting of primary tumors with mouse neonatal stroma results in increased engraftment rate and growth in vivo (10). For this study, 12 localized prostate cancer specimens from men who had not received hormonal therapy were established as xenografts in host mice for 8 weeks; the hosts were then divided into untreated control and castration-treated groups. One of the 12 patient tissues generated sufficient xenografting material to study all stages of androgen depletion and restoration; the remaining 11 patients were analyzed at some, but not all, time points (see table S1 for summary).

Tumor grafts were successfully established from all 12 patients, with an overall inoculation take rate of 64% (60 of 94 grafts from untreated hosts contained malignant foci). Engrafted tissues contained malignant [cytokeratin 8 and 18 (CK8/18)+p63; Fig. 1A] and benign epithelia, accurately reflecting specimen heterogeneity; however, subsequent analyses were only performed on malignant foci.

The pathology of original tumors was retained in engrafted specimens. The Gleason score of the index tumor reported at patient diagnosis and the tumor region acquired for the study were comparable to those in engrafted tissues (table S2). Engrafted tumor foci retained prostate cancer hallmarks including prominent macronucleoli, AR, prostate-specific antigen (PSA), and α-methylacyl-coenzyme A racemase (AMACR) expression (Fig. 1, B to E). Engrafted tumors from 11 of 12 patients contained rare neuroendocrine cells similar to the original specimens (Fig. 1F). However, in one patient specimen (patient 4; table S1), additional neuroendocrine differentiation was present in the original and engrafted specimen, comprising 1.35% of the tumor cells in the xenograft. Engrafted tumors also showed high levels of proliferation and low rates of apoptosis (Fig. 1, G and H). In summary, the xenografts accurately recapitulated features of the original specimens, enabling study of individual patient tissue responses to androgen deprivation and restoration.

**Localized prostate cancer xenografts regress within 3 days of androgen deprivation**

Having established the localized prostate cancer xenografts, engrafted tumor responses to short-term androgen withdrawal were examined by castrating host mice for 3 days (Fig. 2A). Seven patient specimens were committed to this analysis.

In comparison to grafts from untreated controls, the incidence of malignant foci in xenografts from hosts castrated for 3 days was not changed (72% versus 74%). For two patients (2 and 10), the tumor foci in grafts from 3-day castrate hosts were small and insufficient for further characterization. Using the remaining five patient specimens (Fig. 2B), we compared histological analysis of AR and PSA immunolocalization before and after castration. A common decline in AR and PSA staining intensity was observed after 3 days of castration, although across all five patient specimens, there was variation in the remaining immunopositivity (Fig. 2, C and D). Furthermore, quantitation of tumor cell proliferation and apoptosis after 3 days of castration showed evidence of tumor regression. Engrafted tumors responded to 3 days of castration with an overall decrease in proliferation (11.62 to 4.17%; \( P = 0.0014 \)) and an increase in apoptotic index (1.33 to 9.25%; \( P < 0.0001 \)). For each individual patient, the degree of response was variable, and a spectrum of proliferative and apoptotic responses was observed (Fig. 2, E and F).

Overall, these findings show that castration of host mice carrying localized prostate cancer tissues results in regression of the bulk of the tumor tissue, although there is variation in the degree of response.
After prolonged castration, the residual tumor foci appeared atrophied compared to tumors in untreated host grafts (Fig. 3, B and C; shown for patient 1). Similar to untreated tumors, residual tumor cells expressed AMACR and luminal marker CK8/18 but not basal cell markers p63 and high–molecular weight cytokeratin (CKH) (Fig. 3, C and D). Immunolocalization of AR and PSA was absent or markedly reduced in residual tumor foci compared to untreated grafts (Fig. 3, E and F). Similar to the findings in untreated controls, neuroendocrine cells (detected by staining for chromogranin A) were not found in the residual tumor foci after long-term castration (Fig. 3G).

Further characterization revealed that the residual tumor foci were growth-quiescent, showing little evidence of proliferation or apoptosis; formal quantification could only be performed in two patients because the other xenografts did not contain sufficient numbers of cells for analysis (Fig. 3, H and I; patients 1 and 12). This suggests that the active phase of tumor regression was completed, leaving a population of castrate-resistant tumor cells not yet capable of repopulating the tumor in the presence of depleted levels of systemic testosterone.

Other expression markers were used to further characterize the residual foci. Nkx3-1 is a luminal cell marker expressed in all tumor cells before and after engrafting; notably, most of the residual tumor cells were Nkx3-1⁺ (Fig. 3J and fig. S1). Three stem cell markers—CD44, ALDH1, and NANOG—were also detected in the tumor foci but had variable levels of expression across the four patients in both intact and castrated grafts (Fig. 3, K to M, and fig. S1).

To further examine whether castrate-resistant cells preexist in localized specimens, we directly implanted parallel grafts from a further three patients into castrated hosts for 8 weeks. In these grafts, residual foci were identified in 35% of individual grafts (in all three of three patients), although the small clusters of tumor cells observed were insufficient to characterize and quantitate (Fig. 3, N to O). Nevertheless, immunohistochemical analysis revealed that tumor cells that survived in a castrated host also showed low AR and PSA expression, with minimal proliferation or apoptosis, similar to residual foci in grafts that were established in vivo before castration (Fig. 3, P to S). Together, these data demonstrate that in multiple patients with localized prostate tumors, there are preexisting tumor cells with stem-like characteristics that survive castration.

Residual tumor cells are hormone-responsive and can repopulate tumors
To test whether these residual growth-quiescent tumor cells have regenerating potential, we readministered testosterone to castrated hosts bearing three patient specimens (Fig. 4A).

Residual tumor cells were responsive to testosterone, regenerating tumors similar to those in untreated hosts. Tumors were observed in androgen-restored grafts from all three patients (Fig. 4B), even though no foci were visible in two patient-matched grafts before testosterone readministration (table S1). For a third patient, it was possible to perform...
longitudinal analysis of tumors in untreated, 4-week castration, and testosterone-restored hosts (fig. S2).

After testosterone supplementation, tumors displayed AR and PSA immunolocalization similar to that of controls (Fig. 4, C and D, and fig. S2). Two specimens from patients 5 and 6 had rare neuroendocrine cells in untreated grafts, and the regenerated tumors had similar cells (fig. S2). Patient 4, who did have focal neuroendocrine differentiation in untreated tissues, regenerated tumors with 1.24% chromogranin A-positive tumor cells (Fig. 4E). Resumption of tumor growth did not alter apoptosis but significantly increased proliferation compared to untreated controls (Fig. 4, F and G, and fig. S2). These data suggest that cells that persist in localized tumors during castration include cancer cells with regenerative capacity.

DISCUSSION

Here, xenografts of 12 localized prostate cancer specimens were established. The bulk of the tumor cells regressed after castration, but a residual population remained. These cells were growth-quiescent, Nkx3-1+, PSA4, and AR8 and showed variable expression of stem cell
markers CD44, ALDH1, and NANOG. These residual tumor cells were androgen-responsive and repopulated rapidly proliferating tumors upon readministration of testosterone.

The presence of tumor cells in localized prostate cancer specimens that survive castration is an important finding because these residual tumor cells could eventually develop mechanisms to proliferate and/or disseminate in a low-testosterone environment, leading to lethal prostate cancer. The cells that evade androgen deprivation therapy in advanced disease are considered to be therapeutic targets (14), but we are now reporting that such cells exist in earlier stages of prostate cancer.

In our patient cohort, 66% of patient tumors contained residual cancer cells after prolonged castration, and the remaining 33% of specimens were also able to regenerate tumors upon androgen restoration. Immunohistochemical detection of residual foci is not always possible but does not exclude the fact that such foci may be present and functional. Discovery of the genomic and phenotypic characteristics of such residual tumor cells will be essential to design future therapeutic strategies.

Residual tumor cells in localized human prostate tissues expressed markers previously used to identify stem cells in the normal and malignant prostate (8). Residual foci uniformly expressed the luminal marker Nkx3-1+, indicating a CARN-like (castration-resistant Nkx3-1–expressing cells) phenotype (15), and expressed low but detectable AR and PSA. These features of the residual castrate-tolerant tumor cells from localized prostate tumors were similar across multiple specimens but different from the phenotype of the BM18 xenograft line. Castration of BM18 xenografts also revealed stem-like cells with a luminal progenitor phenotype (CARN-like), but the BM18 residual tumor cells also contained a neuroendocrine population (8). Neuroendocrine cells are hypothesized to aid disease progression, particularly the emergence of CRPC (16), but they were not a prominent feature in the localized specimens in our study. This difference could reflect the difference in disease stage because BM18 tumors were originally derived from a bone metastasis and our clinical cohort had only localized disease. Alternatively, neuroendocrine differentiation could be a characteristic of a particular established xenograft line.

The unequivocal evidence of residual tumor cells remaining during castration raises the questions of whether we should target these cells and how to increase the effectiveness of current therapies. The residual tumor cells we identified were able to survive in the androgen-deprived milieu, although the cells remained in a dormant growth-arrested state until testosterone was restored. Proof that these cells are capable of regenerating tumors in the absence of androgens, giving rise to CRPC, requires further investigation. However, these residual cells are likely candidates to give rise to lethal CRPC because they are hormone-responsive, and if adrenal or intratumoral steroidogenesis were to resume, these tumor cells would be expected to repopulate. Hence, there is an incentive to further characterize the potential of these cancer cells to give rise to CRPC and, if appropriate, decipher how to target them before the lethal phenotype ensues.

Unequivocally, there is a clinical need for androgen blockade, but this treatment eventually fails and progression to lethal disease occurs. Adjuvant therapies for high-risk localized prostate cancer patients have previously shown favorable outcomes in the clinic (17), and combined approaches that specifically target these residual quiescent tumor cells may now be justified. This is a provocative finding and provides a considerable challenge as to how to target these specific cells, but doing so earlier in disease may potentially increase the survival benefit for patients. Hence, the model system described in this study should become a valuable preclinical tool, in which potential therapeutic agents can be tested for their ability to target the residual cancer cell population and prevent tumor regeneration in a large cohort of biologically accurate patient xenografts.

An important limitation of this and other studies resides around the selection of tumor tissue for xenografting from the prostatic specimens at the time of resection. Many patients have multifocal disease, and most studies and analyses are conducted at one particular time in disease progression, usually when tissue becomes available at biopsy or surgery. The ability to predict an individual patient’s course of disease...
and/or response to treatment is restricted by the selected tumor foci used for xenografting. The means to identify or predict which tumor foci in a patient specimen will be the foci that evade all treatment leading to metastasis and death is completely unknown. Thus, studies that chart the longitudinal progression of disease in each patient and the characteristics of each tumor foci at each stage of treatment, ending in lethal CRPC disease, are necessary. At present, tissue selection is a limitation of this and other xenografting studies because there is an untested hypothesis that not all tumor foci are equal and equally lethal, although the evidence to support that notion remains to be provided.

In conclusion, in an experimental model for engrafting localized prostate cancer specimens, a subset of tumor cells persisted during long-term castration and regenerated the tumor upon readministration of testosterone. These results show that residual populations of growth-quiescent, stem-like tumor cells preexist in localized prostate cancer specimens before the onset of CRPC. Future characterization of these residual tumor cells is required to further evaluate their potential role as precursors to CRPC and adjuvant therapy targets. Ultimately, successful eradication of potential precursors to the lethal CRPC phenotype would maximize the effectiveness of androgen deprivation therapy and lead to improved clinical outcomes for men with prostate cancer.

MATERIALS AND METHODS

Study design
The sample size for each experiment, including treatment type or time point for analysis, was dependent on the amount of tissue available for xenografting, which varied for each patient specimen. Experiments were designed to include six patient samples per group, but in some cases, analysis was restricted to three patients per group. Host mice carrying xenografts were randomly and equally assigned to either control or treatment groups. Pathological assessment of tissue sections was performed in a blinded manner with coded labels, but all other immunohistochemical and stereological analyses were open to the investigators.

Animals
Seminal vesicle mesenchyme (SVM) was used to stromal-support xenografts of human prostate cancer tissues; we previously showed SVM to be equivalent to urogenital mesenchyme (UGM) in inducing prostatic differentiation (10, 18). SVM was isolated from day 0 BALB/c or C57BL/6 male mouse pups as previously described (Monash Animal Services) (19). Graft hosts were male nonobese diabetic/severe combined immunodeficient (NOD/SCID; Animal Resources Centre) and NOD/SCID γ (NSG) mice (from our own colony). Animal use and procedures were approved by the Monash University Standing Committee of Ethics in Animal Experimentation (SOBSA 2010/67).

Human tissues
Twelve localized tumors were obtained from radical prostatectomy patients with informed consent after explaining the nature and possible consequences of the studies. Human subject research was approved by Cabrini Human Research Ethics (03-14-04-08), Epworth Hospital (34306), and Monash University (2004/145). Prostatectomy specimens were transported to the pathologist on ice, who dissected out a tumor region for the study, which was then transported in RPMI 1640 to the laboratory. Specimens were cut into ~4-mm³ pieces and either fixed in formalin or stored in RPMI/10% fetal calf serum/testosterone (10⁻⁸ M)/insulin (10 μg/ml)/hydrocortisone (10 μg/ml) at 4°C until xenografts were prepared.

Xenograft preparation
Tumor pieces were prepared with ~250,000 mouse SVM cells, as previously described (18, 20), to support the survival and growth of the tumors in vivo (10). Prepared grafts were implanted under the kidney capsule of host NOD/SCID or NSG mice for 8 to 16 weeks. Host mice were supplemented with a 5-mm testosterone implant, except for mice that were castrated before grafting surgery.

Castration of host mice
Grafts were established in host mice for about 8 weeks before half the hosts were castrated and underwent removal of testosterone implants to model androgen deprivation therapy. For androgen restoration studies, castrated mice were supplemented with a 5-mm testosterone implant. Three patient tissues were inoculated into hosts that were castrated 1 week before grafting and established for 8 weeks. A summary of experiments conducted on patients and tumor take rates can be found in table S1.

Harvest of xenografts
Harvest occurred at 3 days or 4 weeks after castration or 4 weeks after testosterone restoration. All studies had some grafts that remained in untreated control hosts. Grafted tissues were measured, weighed, and fixed in 10% formalin for 24 to 48 hours before being processed and embedded in paraffin.

Immunohistochemistry
Immunohistochemistry was performed on a Leica BOND-MAX automated system (Leica Microsystems Pty. Ltd.) or manually with the DAKO EnVision+detection system. Primary antibody details and immunohistochemistry conditions are detailed in table S3.

Stereology
Uniform systematic random sampling was used to estimate the percentage of Ki-67−, caspase-3−, and chromogranin A−positive tumor cells in xenografts with the newCAST component (version 2.14; Visiopharm) of Visiopharm Integrator System (version 2.16.1.0; Visiopharm) (21). Two sections for each graft were analyzed. Only grafts that contained sufficient malignant foci for unbiased sampling were analyzed. Analysis was conducted on 25% of each section.

Statistics
Prism 5 software (GraphPad) was used for all analyses. Percentages of Ki-67 and caspase-3 were compared with Student’s t test. Data are expressed as means ± SEM.

SUPPLEMENTARY MATERIALS
www.sciencetranslationalmedicine.org/cgi/content/full/5/187/187ra71/DC1
Fig. S1. Expression of stem cell markers in residual tumor cells during long-term androgen deprivation.
Table S1. Summary of patient tissue distribution in the study and survival of tumor foci in xenografts.
Table S2. Gleason pattern of established xenografted tumors compared to the original specimens.
Table S3. Antibody conditions for immunohistochemistry.
REFERENCES AND NOTES


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A Preclinical Xenograft Model Identifies Castration-Tolerant Cancer-Repopulating Cells in Localized Prostate Tumors

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The Enemy Within

Prostate cancer is one of the most common types of cancer in men. In advanced stages, it is typically treated with medications that mimic castration, depriving the tumor of androgen stimulation. Unfortunately, these cancers eventually become castration-resistant and begin to grow even in the absence of hormonal input. What isn't known is how these cancer cells develop the ability to survive androgen deprivation, and whether some types of stem-like castration-resistant cells are already present in prostate cancer from early stages or evolve later during the course of treatment. Now, Toivanen and colleagues shed some light on this mystery, with a report of castration-tolerant cells derived from early localized tumors that had not yet been exposed to anti-androgen therapy.

The authors used primary prostate tumors from 12 men with localized cancer, implanting them in a mouse xenograft model to study the effects of androgen deprivation on the tumors' survival. Castration of the host mice led to rapid regression, but not disappearance of the tumors. Even after a prolonged period of castration (4 weeks), some residual tumor foci persisted. When testosterone stimulation was restored in the host animals, these residual cells rebounded, regenerating masses that were histologically similar to the original tumors.

This work by Toivanen et al. indicates that some prostate cancer cells can survive castration and later repopulate the tumor when androgen stimulation is available. Thus far, there is no indication that these castration-tolerant cells can proliferate in the absence of androgens, unlike the cells found in more advanced "castration-resistant" prostate cancer. Additional work will be needed to clarify whether these might be a type of prostate cancer stem cells, and what makes them different from the population of "androgen-sensitive" cancer cells that do not survive androgen depletion. Although there are many questions that must still be answered about the biology of these castration-tolerant cells, this work raises the intriguing possibility that we may eventually be able to specifically target and eradicate them, thus preventing prostate cancer recurrence in patients.