Advances in prostate cancer research models: From transgenic mice to tumor xenografting models

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Received 21 October 2015; received in revised form 1 January 2016; accepted 2 February 2016
Available online 2 March 2016

Abstract The identification of the origin and molecular characteristics of prostate cancer (PCa) has crucial implications for personalized treatment. The development of effective treatments for PCa has been limited; however, the recent establishment of several transgenic mouse lines and/or xenografting models is better reflecting the disease in vivo. With appropriate models, valuable tools for elucidating the functions of specific genes have gone deep into prostate development and carcinogenesis. In the present review, we summarize a number of important PCa research models established in our laboratories (PSA-Cre-ERT2/PTEN transgenic mouse models, AP-OX model, tissue recombination-xenografting models and PDX models), which represent advances of translational models from transgenic mouse lines to human tumor xenografting. Better understanding of the developments of these models will offer new insights into tumor progression and may help explain the functional significance of genetic variations in PCa. Additionally, this understanding could lead to new modes for curing PCa based on their particular biological phenotypes. © 2016 Editorial Office of Asian Journal of Urology. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second most common cause of cancer-related mortality among males in the United States, with a lifetime risk of one in six [1,2]. Androgen-deprivation therapy (ADT) is the first-line therapy to improve survival and reduce morbidity in primary or metastatic PCa. Current measures for combating the disease are partially effective, but they are usually not specific for PCa, causing unwanted side effects. The side effects can be serious and leave the possibility for recurrence in a more aggressive, androgen-independent form [3]. Further treatments for castration-resistant prostate cancer (CRPC) after ADT remain a challenge because patients’ performance status often progressively declines [4]. Although researchers have identified defined histological alterations in PCa, the nature of this genetically heterogeneous disease has restricted the identification of novel alternatives of genes that can be used as therapeutic targets [5].

The prostate is a canalized ductal–acinar structure that develops from the embryonic urogenital sinus (UGS) to form a predominantly fibromuscular phenotype [6]. The ductal–acinar structure is constituted with tall columnar secretory luminal cells and a flattened basal epithelium [7,8]. The expression of cytokeratins (CK) and characteristic biomarkers are distinct in prostate basal and luminal epithelial cells. Basal cells express CK5, CK14 and tumor suppressor p63, whereas luminal cells express CK8, CK18 and the androgen receptor (AR) as well as prostate-specific antigen (PSA) [9,10]. Other basal cell markers including B cell lymphoma/leukemia-2 (BCL-2), epidermal growth factor receptor (EGFR), and mesenchymal to epithelial transition factor (MCT) have also been reported [11–14]. Prostate luminal and basal epithelia cells can be generated from stem cells [15]. Luminal cells have long been considered as the cellular origin of the majority of PCa. However, recent studies suggested that prostate basal stem and progenitor cells can also give rise to PCa [16–20]. It has been proposed that mature luminal cells and basal cells are developed independently and that luminal cells are derived from self-renewing luminal stem cells [21,22]. Based on several mouse studies, some have proposed that fully differentiated luminal cells can display benign or malignant proliferate characteristics in vivo [23], but the mechanisms responsible for prostate luminal epithelial colonial proliferation and regeneration remain unclear.

Many oncogenes and tumor suppressor genes are mutated across a large extent of PCa, such as Ras, Myc, p53 and phosphatase and tensin homolog deleted on chromosome ten (PTEN) [24–28]. Although these genes are frequently altered in PCa, their role in tumor initiation and progression is not known. However, most PCa contain a usual set of “driver mutations”, thus unveiling multiple oncogenic routes that are highly dependent on the disruption of specific pathways such as p38/MAPK, Notch and PI3K/Akt [29–35].

The investigation of human prostate models has yielded a better understanding of prostate oncogenesis and cellular differentiation. A few potential prostate models have been established for how oncogenic disruption of particular differentiation pathways can promote tumor initiation [36,37]. Some estimate that the cells of origin are not committed luminal or basal stem cells in PCa, but rather a transient-intermediate cell, which is found in both human and mouse prostate [38]. Accordingly, a deeper understanding of the cell of origin in PCa with appropriate prostate models is needed, and being able to find more sensitive ways to detect prostate tumors and predict tumor aggressiveness is also a challenge.

Prostate stem and progenitor cells are defined by their ability to undergo self-renewal and multipotent differentiation. It is important to develop some models of prostate stem and progenitor cells for understanding the molecular mechanisms of prostatic development, maturation, and malignant transformation [9,39]. In recent reports, scientists established relevant in vivo models that examined basal-derived cells for prostate regeneration [40]. Additionally, there are few available cell lines that could inform prostatic biological status and that can be used to examine prostate carcinogenesis. As a consequence, there is an instant need for prostate cell lines that recapitulate the different phenotypes identified in actual human tissue samples. Tissue recombination is a valuable instrument for analyzing the functional remodeling of human prostatic tissues in immunodeficient mouse models [41]. Unfortunately, most of the present studies lack understanding of the detailed mechanisms driving PCa. In this review we describe several established PCa models that could potentially drive new specific therapeutic agents for increasing efficacy and reducing side effects.

2. Transgenic mouse models: PSA-Cre-ERT²/PTEN mouse line

The identification of specific cancer biomarkers, such as Ras, Myc, p53 and PTEN, lie in the center of current challenges in the recognition of tumor initiation, prediction of prognosis, and design of targeted therapies. Down-regulation of PTEN was first recognized in the late 1990s in about 10% of primary prostate tumors and in 65% of metastatic tumors [42–44]. There is now abundant information illustrating down-regulation of PTEN via mutation, transcriptional repression, or deletion in many cancers, including PCa [45,46]. PTEN is one of the most common genetic transformations that can regulate PI3K/Akt pathways to influence cellular function [47,48]. It was also reported that the down-regulation of PTEN was associated with poor prognosis in PCa patients [43,49,50]. Notably, many people concluded that the tissues from castration-sensitive patients expressed high PSA, the marker of differentiated luminal cells. Following injection of immunodeficient mice, both PSA-negative and PSA-positive cell populations displayed tumor-initiating capability, but the PSA-positive population generated more and larger tumors [51].

Due to the lack of in vitro models for studying human oncogenesis, the translation of these genetic modifications in the mouse to understand the diseases of prostate are
highly informative. Mouse models are helpful for genetic experiments without the same ethical limitations as humans [52,53]. Transgenic mouse models rely on cyclization recombination enzyme (Cre) activity via "cell-specific" promoters, such as K5, K8 or Nkx3.1 [21,54]. It also has been reported that specific expression of bacteriophage P1 site-specific Cre recombinase is the key for formation of conditional gene targeting in mouse. A DNA segment, flanked by two Lox P sites (floxed DNA), can be excised efficiently during the Cre recombinase [55,56]. The reproduction of transgenic animal models for human diseases in different organs depends on the efficient introduction of the mutations in a gene over a period of time [57,58]. With conditional knockouts, we can avoid embryonic lethality with conventional gene knockout techniques. Choi et al. [25], for example, established inducible Cre-LoxP-mediated genetic cell-lineage tracing to characterize the differentiation potential of luminal and basal progenitors in adult mouse prostates [54,59]. In the same paper, they argued that both basal and luminal cells were unipotent and self-sustained lineages which could generate independent epithelial cell types in adult mouse prostate. More importantly, they demonstrated that PTEN knockdown in either the basal or luminal cell lineages could lead to the occurrence of PCa exhibiting different susceptibility. In the another article [60], they reported success in the generation and detailed molecular characterization of seven human-derived PCa organoid lines derived from diverse disease sites including circulating tumor cells (CTC). These lines harbor copy number signatures of primary PCa, including SPOP mutation, PTEN loss, TMPRSS2-ERG interstitial deletion, as well as alterations commonly found in CRPC, including TP53, PIK3R1, FOXA1 and several chromatin modifier mutations. Better understanding of the functions of specific genes in PCa will be required to validate suitable transgenic mouse models.

In our previous studies, we reported that a 6.0-kb PCR amplified DNA fragment containing three androgen response elements (ARE), one enhancer and the proximal promoter of human prostate-specific antigen (hPSA) gene was cloned into the Sall site of pGS-Cre-ER<sup>T2</sup> to generate pPSA-Cre-ER<sup>T2</sup> [61,62]. The DNA fragment is fused to the Cre-ER<sup>T2</sup> recombinase, which is fused to a mutated ligand-binding domain (LBD) of the human estrogen receptor (ER) containing the G400V/M543A/L544A triple mutation [62]. These transgenic mice mimic the expression of the human endogenous androgen-regulated PSA gene with specific Cre recombinase in prostate luminal epithelial cells [19,63]. PSA-Cre-ER<sup>T2</sup> mice were cross-bred with floxed homozygous PTEN mice which have been described in Suzuki's research [10] to generate double transgenic PSA-Cre-ER<sup>T2</sup>/PTEN mice. Tamoxifen was injected ip daily for 5 consecutive days (D1–D5) to 8-week-old mice [64,65]. At this point, conditional PTEN floxed alleles were generated in the mice, and the expression of PTEN was subsequently ablated through breeding with transgenic mice which express the Cre recombinase under the control of the PSA promoter [66]. The generation of PSA-Cre-ER<sup>T2</sup> mice allows us to target floxed genes selectively in prostate luminal epithelial cells and to coordinate the number of epithelial cells that are genetically altered. Our PSA-Cre-ER<sup>T2</sup>/PTEN mouse model with ablated tumor suppressor gene PTEN closely duplicates the progression of human PCa. In a previous study, after PTEN ablation, we found the prostate epithelium displayed significant cytologic atypia in 4 weeks and prostatic intraepithelial neoplasia (PIN) in 2–3 months. It is noteworthy that the two kinds of precancerous lesions usually occur in the dorsolateral lobe, which is the most similar genetically to the human prostate. After 10 months, some precancerous lesions begin to form tumors [66]. In another report using PSA-Cre-ER<sup>T2</sup>-based genetic lineage marking/tracing in mice, preexisting luminal epithelial cells were shown to be a source of regenerated luminal epithelial cells in the adult mouse prostate. That study demonstrated the survival and proliferation of luminal epithelial cells in response to castration and androgen replacement in transgenic mouse models [67].

According to these results, we can assume that the PSA-Cre-ER<sup>T2</sup> transgenic mouse will be a valuable tool for clarifying the functions of particular genes in prostate development and carcinogenesis, and also an important measure to study preventive and therapeutic approaches in vivo. This model represents a clinically relevant model of PCa development and progression.

In some aspects, investigations in transgenic mice are limited. For example, signaling mechanisms for differentiation in human and mouse prostate epithelial cells may differ. Our PSA-Cre-ER<sup>T2</sup> transgenic model, showed no distant metastases even after PTEN ablation for extended periods, indicating that progression to metastasis requires an additional mutation or mutations [66]. Taken together, the present data show that our transgenic mouse models produce the initial phases of progression of human PCa.

3. Human prostate cancer-mouse anterior prostate (AP)-orthotopic xenografting (OX) model (AP-OX)

It is widely recognized that the majority of PCa deaths are due to the tumor metastasis, especially skeletal metastases [68], but modeling this process in mice has proven difficult. Furthermore, PCa induces an osteoblastic reaction within the bone, which is rarely observed in other tumors. What causes bone metastasis and osteoblastic lesions in PCa remains unclear. The incurability of PCa is not only related to the tumor itself, but also to the interactions between tumor cells and their microenvironment. This tumor microenvironment produces various cell types, growth factors and cytokines, and numerous extracellular matrix (ECM) components. The interactions between tumors cells and their microenvironment are required for invasion, angiogenesis, and metastasis to other organs [69,70]. Factors in the microenvironment can promote epithelial-mesenchymal transition (EMT) via up-regulation of specific transcription factors. The cancer cells present mesenchymal phenotypes by EMT programming and then cleave the ECM, exit the tumor microenvironment and intravasate into blood vessels to travel to distal organs [71]. Much attention has been given to in vivo animal...
experiments to research the PCa microenvironment. A number of preclinical mouse models of PCa are currently available, including many transgenic mouse models and fewer orthotopic xenografting mouse models [72,73]. Orthotopic prostate xenografting mouse models produce more heterogeneous cohorts of tumors and controlled approaches than transgenic mouse models [74]. Human PCa orthotopic implantation into the prostate of immunodeficient mice has been proven as a vital method for PCa research.

For the orthotopic xenografting model, subconfluent luciferase-expressing tumor cells such as PC3 or LNCaP are mixed with neutralized collagen gel, implanted into the mouse anterior prostate (AP) lobe through a lower midline laparotomy incision, and then injected subcutaneously into the right flank of 10-week-old nude male mice [74–76]. After xenografting, tumor growth is detected by whole-animal bioluminescent imaging performing using an in vivo imaging system (IVIS) biweekly [77]. Additionally, osseous metastases are monitored by X-ray in these orthotopic xenografting models [76].

The AP-OX models in vivo have been successfully used for studying the biological functions of some genes involved in metastasis in PCa. Hafeez et al. [75] demonstrated that the importance of Plumbagin (PL) for cancer cell growth, invasion and metastasis using an AP-OX model. Xiang et al. [77] presented that SPARCL1 decreased invasive and metastatic progression significantly in OX models. Additional studies of these tumour-related genes will be valuable for determining its mechanisms of metastasis and generating potential anti-metastatic agents for the treatment of PCa. Alternatively, AP-OX models are of great benefit for the study of the curative effects of novel clinical trial drugs on tumor cell proliferation and regional lymph node metastasis particularly [74]. This new model better recapitulates the clinical situation, adding significance to the study of the biological characteristics of bone-metastatic PCa and for exploitation of specific treatments.

Human prostate cancer-mouse AP-OX models, in contrast to transgenic mouse models, provide a beneficial alternative approach for understanding the specific interactions between various molecularly and genetically altered tumor cells and the tumor microenvironment [72,78–80]. These human tumor xenografting models retain the growth and histopathological features characteristic of the original cancers and have been used for rapid screening of potential therapeutics. In addition, orthotopic implantation united with subsequent harvesting at metastatic sites can draw upon mutations of tremendous clinical relevance to the metastatic process [81]. In comparison to ectopic subcutaneous tumor models, the OX models can more precisely reconstitute a tumor microenvironment that influences the phenotypes of tumor cells, as originally proposed by Stephen Paget’s “seed and soil” hypothesis and affirmed by numerous others [82]. In summary, human prostate cancer-mouse AP-OX greatly complement transgenic mouse models, providing valuable tools to study PCa progression more deeply.

Unfortunately, some human tumor xenografting models are associated with exceedingly low tumor take rates and only successful in case of highly advanced malignancies [83]. These deficiencies reduce the usefulness of such models for studies of PCa metastasis and restrict the predictive power of such models with regard to patient responses to anticancer drugs in the clinic.

4. NHPrE1- and BHPRE1-based tissue recombination-xenografting model

The mechanisms of self-renewal and multipotent differentiation in prostate stem and progenitor cells are important to understand the molecular mechanisms of prostate development as well as cancer initiation and maturation [9,39]. It is also important to understand the role of inflammation on benign growth and tumorigenesis of human cells [23,84]. However, there are not enough stable benign cell lines available to verify the function of stem and progenitor cells in prostatic development and tumor initiation. Accordingly, the development of prostate epithelial cell lines that adequately recapitulate benign histology as well as the various tumor phenotypes is sorely needed. Tissue recombination is a valuable tool for studying the functional remodeling of human prostate [85]. In recent years, immortalized human prostate epithelial (HPrE) cell lines including BPH-1 have been reported to recapitulate the functions of human prostatic tissues [86]. However, BPH-1 cells often demonstrate squamous hyperplastic differentiation in tissue recombinants and inaccurately recapitulate prostatic development [87].

To meet this challenge we developed two novel spontaneously immortalized cell lines from adult non-tumorigenic human prostate epithelium, NHPrE1 (CD133high/CD44high/OCT4high/PTENhigh) and BHPRE1 (p63high/p53high/p21low/WAF1high/RBhigh). NHPrE1 cells were characterized as a putative progenitor cell line, and BHPRE1 cells were characterized as a putative epithelial intermediate cell line [88].

Recombination of human prostate epithelial stem cells with rat embryonic urogenital sinus mesenchyme (UGM) functionally re-establishes the stem cell niche and allows for the assaying of stem cell properties in vivo. The structures and phenotypes of the recombinants made with our spontaneously immortalized cell lines depended on the ratio and nature of implanted epithelial cells [89] and relied on UGM similar to previous reports [85].

The NHPrE1 and BHPRE1 cells are able to regenerate benign secretory ductal–acinar architecture in vivo, which contains both basal and luminal epithelial cells expressing appropriate CK profiles [90–92]. Because the NHPrE1 are more of a progenitor cell, regeneration usually only needs a minimum of 10 cells, whereas the more intermediate BHPRE1 cells required at least 200 000 seeding cells. It was noted that the human prostatic biomarkers including PSA, Nkx3.1, androgen receptor (AR), and 15-lipoxygenase-2 (15-LOX-2) were expressed in the regenerated epithelia appropriately [88]. Accordingly, the NHPrE1 and BHPRE1 cell lines represent potentially significant tools in which to investigate the mechanisms associated with human prostatic regeneration, pathogenesis, and carcinogenesis. As
such, these cell lines represent potentially useful models in which to start to investigate mechanisms associated with both benign and malignant disease.

5. Prostatic organoid culture

There have been unprecedented developments in the utilization of human tissue surrogates in vitro during the past years. Despite many attempts by numerous investigators, however, it has been difficult to increase the number of available cell lines in public cell line repositories with less than 10 for PCA [93,94]. The under-representation of PCA cell line models for research stems from the difficulty in propagating tumor cells for a long time in vitro. In order to represent the spectrum of clinical genotypes of PCa, new cell lines which display the observed clinical phenotypes are urgently required [95]. Adult stem and progenitor cells can be embedded in a special three-dimensional (3D) matrix without stroma and allowed to self-organize. Such ‘3D’ culture systems not only contain analogs of ECM, but also mix with some conditions that enhance the differentiation, proliferation and survival of stem or progenitor cells [96]. The generated organoids represent the biological characteristics of native epithelium much better than the traditional PCA cell lines [97].

The pseudostratified epithelium in the prostate gland consists of basal and luminal cells. In the tissue recombination-xenografting models mentioned above, the basal cells reconstitute a whole prostate gland and luminal cells can generate basal cells. The molecular details of these transitions and whether they occur in humans remains unclear. Prostate organoids in a 3D culture system confirmed that both basal and luminal cells could generate a complete multilayer prostate organoid and showed luminal cells could generate both basal and luminal lineages [98]. Organoids generated from tumor or normal prostate epithelium reveal adenoid architecture containing luminal and basal cells, undergo expansion and express AR [99]. Organoids are genetically stable and controllable, and can be applied to mechanistic studies as a luminal multilinage progenitor cell model.

The crucial breakthrough, however, is the optimization of culture media that allows the infinite proliferation of both benign and malignant prostate cells, maintains genetic stabilization without drift and improves the generation of new cell lines with a higher success rate [98]. The two ways for prostate organoid culture of isolated prostate epithelial cells are floating in low-percentage Matrigel and allowing self-organization. Such ‘3D’ culture systems not only contain analogs of ECM, but also mix with some conditions that enhance the differentiation, proliferation and survival of stem or progenitor cells [96]. The generated organoids represent the biological characteristics of native epithelium much better than the traditional PCA cell lines [97].

6. Patient-derived xenografting (PDX) model

Preclinical models for drug trials are normally grounded in immunodeficient mice carrying PCA cell line xenografts,
as mentioned above. Unfortunately, the increasing homogeneity of established cell lines after long-term culturing in vitro was observed, resulting in failure to regenerate clinically-relevant heterogeneity [107]. Additionally, cell line-based xenografts rarely display the organizational architecture of the original prostate malignancies and, consequently, do not accurately recapitulate the intricate interactions between the PCA cells and the tumor microenvironment [108]. Standardized and representative preclinical models that recapitulate the dynamics of PCA treatment are urgently required. In theory, PDX models, based on direct transplantation of fresh tumor specimens from PCA patients subcutaneously, orthotopically or under the kidney capsule of immunodeficient mice (e.g., severe combined immunodeficiency (SCID) mice, NOD scid gamma (NSG) mice), meet the clinical demand [109].

PDX models have been used for the preclinical investigation of various aspects of PCA including angiogenesis, identification of castrate-resistant stem-like cells, effects of anti-androgen therapies, and interactions between tumor cells and the bone microenvironment [110–114]. At the histopathological level, the PDX models maintain, especially initially, the stromal components and tissue architecture of the original tumors and are considered an accurate representation of the complex biochemical milieu in PCA [111,115]. At the cellular level, PDX models also sustain inter-tumoral and intra-tumoral heterogeneity, as well as the molecular characteristics of the original tumors, including gene expression profiles [107,116–119], chromosomal copy number variants [116,120,121], and single-nucleotide polymorphisms [117,122]. In clinical practice, PDX have been used to predict and confirm drug responses [123], exploit biological markers for standard and novel antineoplastic drugs [111], and estimate the therapeutic effects of patients [124]. Unfortunately, PDX are unfit for in vitro cultures in regard to initial high throughput drug screens [125]. Recently organoid culture has increased the available preclinical tumor models by narrowing the gap between cell lines and xenografts [126]. PDX provide a prominent opportunity to capture some of the diversity, complexity and therapeutic responsiveness of clinical PCA [99,127]. Features such as a spectrum of histological characteristics, responsiveness to androgens and relevant chemosensitivity enable preclinical modeling of the disease. Furthermore, continuously updated implements that may provide the ability to distinguish human and murine cells at the histological [128] or gene expression level [129] provide interesting opportunities to determine the contribution of the tumor cell and host stroma to the pathobiology underlying PCAs.

Primary PCA samples were obtained from histologically proven patients with different stages and therapeutic results. The xenografts were derived from both androgen-dependent and androgen receptor-negative castrate-resistant PCA specimens [130,131]. Using xenograft techniques, patient specimens were transplanted into SCID mice [83]. In brief, for sub-cutaneous transplantation, the fragmented tumor sample was mixed with high concentration Matrigel at the same volume and 0.1 mL was injected. For intra-femoral injection, the minced samples were disaggregated by digestion in Accumax, filtered through mesh filter steriley, and then mixed with Matrigel. After that, a 15 µL mixture with about 50 000 cells was injected [110]. To detect the growth of nascent tumor, serum PSA, caliper measurement and other experimental methods were used weekly [132]. The PDX models are more accurate than cell line derived xenografts because they preserved the highly genotypical and histopathological characteristics of the original clinical samples [83,133].

However, similar to other PCA models in vitro, PDXs have their inherent limitations and deficiencies. Firstly, tumor—host interactions are not always conserved across species and functional human immunity is mostly absent in host mice [134]. To overcome the shortcoming, more sophisticated humanized models should be exploited by co-grafting of tumor tissue along with bone marrow stem cells of the same patient simulating the humanized immune systems in mice [135,136]. Secondly, soaring expenses and abundant human resources, compared to traditional cell line-based systems, are imminently required to promote the widespread use of PDX models [137]. Widespread use of PDX models is limited by the time and cost required to generate these models. It can take between 4 and 8 months for detectable tumor growth in mice [138]. Most notably, development of PDX models has been hampered by low success rates when grown under standard tissue culture conditions in vitro, mostly on account of poor vascularization in the transplantation site [139]. However, these disadvantages are counteracted by the outstanding clinical relevance of the PDX model, as it is a most critical requirement for cancer models in drug efficacy and predictive biomarker development studies [109,111].

7. Summary
In conclusion, a complement of transgenic mouse lines and human tumor xenografting models are necessary to appropriately address individual components of PCA initiation and progression (Table 1). While models in vitro allow a more convenient and detailed analysis of cancer-related pathways, it is becoming increasingly evident that the use of different models may apply to the research of different steps of tumor progression. The development of better animal models that fully recapitulate the molecular events as seen in human PCAs is paramount to deciphering tumor progression [140]. Clearly, a better pre-clinical balance between improved in vitro mimicry and selecting models that are more closely related to the clinical scenario is urgently needed. Only through a better understanding of oncogenesis can we find new methods to classify prostate tumors and more accurately predict tumor aggressiveness. We must develop PCA-related mouse lines or models that could display comprehensive disease progression, and be used for effective therapy development and testing. The discoveries based on these models would ultimately provide an optimum clinical perspective to conquering this widespread disease.
Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

The study was supported by funding from the NIDDK (DK098277) to Douglas W. Strand, and from the National Nature Scientific Foundation of China (NSFC No. 81372772) to Dr. Ming Jiang, the Scientific Research Foundation for Jiangsu Specially-Appointed Professor (Sujiaoshi [2012] No. 34), to Dr. Ming Jiang, Department of Education in Jiangsu Province, China and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), China.

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Table 1

| Models | Transgenic mouse lines: AP-OX PSA-Cre-ERT2/PTEN |
|--------|-------------------------------------------------|
| Genes  | PTEN                                            |
| Research direction | Genetic experiments | Tumor microenvironment |
| Application | 1. Clarify the functions of particular genes in prostate development and carcinogenesis 2. Study preventive and therapeutic approaches in vivo |
| Limitations | No distant metastases even after PTEN ablation for extended periods |
| Representative References | [66,67] [75–77] [88] [98,99] [111] |
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