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ANIMAL MODELS OF HUMAN DISEASE

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Animal Models of Human Disease

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Preface

Various human diseases caused by genetics and environmental factors significantly impact the quality of life of the patients and their families, as well as impose a heavy economic burden on the society. We believe that the ultimate goal of understanding human biology is to prevent and cure diseases, and to alleviate pain and suffering, thus allowing individuals to maintain a healthy and active lifestyle. The sophisticated biological processes and complexity of the human body made it challenging for scientists to elucidate the underlying molecular and cellular mechanisms of various diseases. This is further complicated by ethical concerns and difficulties in obtaining proper tissue samples at various stages of disease progression.

The focus of this volume of Progress in Molecular Biology and Translational Science is on animal models of human diseases. Many biological processes and signaling pathways are evolutionarily conserved between animals, including humans. With the powerful genetics and ease of manipulation, animal models have greatly facilitated our understanding of the basic molecular and cellular mechanisms underlying various complex biological processes and human diseases. Here, we highlight the use of animal models to study various disease pathogenesis and their contribution to therapeutic development. We present some of what we believe to be the most common health issues faced by the general public, including cancer, cardiovascular, eye, metabolic, and neurological diseases. In addition to mouse models, this volume has included, when possible, chapters on vertebrate and invertebrate models such as zebra fish and Drosophila that are currently used by scientists to model the respective diseases.

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Modeling Human Prostate Cancer in Genetically Engineered Mice

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The progression of prostate cancer is a slow and multiple-step process; clinically detectable prostate cancer normally manifest in aged men, although the lesions may have originated much earlier in life. Animal models that mimic the initiation, progression, and metastasis of human prostate cancer are needed to understand the etiology of prostate cancer and to develop new treatments. Recent progress in mouse genetic engineering technology has led to generation of a series of mouse models for prostate cancer research, which have been widely used for testing impacts of a single or combinations of several gene alterations on the onset, progression, and metastasis of prostate tumors, as well as for assessing the effects of environmental, clinical, and preclinical drugs for prostate cancer prevention and treatment. Although it is possible that no single "perfect" model can recapitulate every aspects of this highly heterogeneous disease, it is expected that the models mimicking certain aspects of prostate cancers will continue to provide preclinical guide to treat this prevalent disease.

I. Introduction

The prostate is an accessory gland of the mammalian male reproductive system, which produces prostatic fluid that contributes to 25–30% of the volume of the semen. Its morphology varies considerably among mammals. Human prostate is an acorn-shape gland that sits under the bladder and in front of the rectum. Adenocarcinoma of the prostate has become the most common cancer in American men; more than 217,000 new cases are diagnosed every year in the USA alone. Prostate cancer is responsible for more than 32,000 deaths in America per year; the mortality rate is second only to lung cancer.[1] The human prostate has three histologically distinct regions: the peripheral zone, the transition zone, and the central zone. About 85% of human prostate cancer arises in the peripheral zone, whereas benign prostatic hyperplasia (BPH), a nonmalignant overgrowth that is fairly common among aging men, occurs mainly in the transition zone.[1] The progression of prostate cancer is a slow and multiple-step process. Early prostate tumor is organ-confined, responsive to androgen deprivation, and is often surgically curative. At advanced stages, however, the tumors frequently metastasize primarily to bones, lymph nodes, and the lung and become castration resistant. Metastatic prostate cancer usually is lethal, and there is still no cure for men with this advanced disease. Although prostate cancer may originate as localized lesions early in life, most prostate cancer patients may have clinical symptoms only after they are over 60 years of age. Therefore, animal models that mimic the processes of onset, progression, metastasis, and escape from hormone therapies in human prostate cancer are needed to develop new therapeutic strategies for prostate cancer prevention and intervention.

Unlike human prostate that has an acorn-shaped morphology, rodent prostates have four pairs of lobes: the anterior prostate (AP, also known as the coagulating gland), dorsal prostate (DP), lateral prostate (LP), and ventral prostate (VP) lobes. The dorsal and lateral lobes are often collectively referred to as the dorsolateral prostate (DLP) lobes. These morphologically and histologically distinct lobes are arranged circumferentially surrounding the urethra. They display characteristic patterns of ductal network and produce lobe-specific sets of secretory proteins. Although the human and rodent prostates are morphologically different, they have an overall similar histology structure, which consists of epithelial and stromal compartments separated by basement membranes. Furthermore, histology and gene expression similarities between human peripheral zone and the rodent DLP suggest that they are structurally and functionally equivalent.[2,3]

Despite the differences in details of organ morphology and tissue histology, mouse and human prostates share extensive similarities in basic cellular and molecular biological features (Fig. 1). The rodent and human prostates have overall similar intimate two-way regulatory communications and symbiosis between epithelial and stromal compartments. Similar to human prostate, the mouse prostatic epithelium has three major cell types, luminal cells, basal cells, and neuroendocrine (NE) cells. These three major cell types can be distinguished by their morphological characteristics, molecular markers, secretory proteins, and relevance to progression of prostate tumors. Luminal cells are the predominant cell in the prostate epithelium, which are androgen-dependent and produce secretory proteins. The luminal epithelial cells are characterized by the expression of the androgen receptor (AR), cytokeratins 8 and 18, and the cell surface marker CD57, which are also exhibited in most human prostate cancer cells. The basal cells are located between the luminal cells and the basement membrane, which are characterized by the expression of cytokeratin 5, cytokeratin 14, CD44, and p63. Although some basal cells are AR positive, most basal cells do not express the AR. Although the function and the cell lineage of basal cells remain controversial, evidence reveals that epithelial stem cells and transient amplifying cells of the prostate reside in the basal cell compartment.[4,5] The NE cells are a minor population of uncertain embryological origin believed to provide paracrine signals to support the growth of luminal cells.[6,7] NE cells can be identified by expression of synaptophysin, chromogranin A, and synaptic vesicle protein 2. Regardless that most prostate cancer exhibits luminal epithelial markers and loses basal cell markers, both luminal and basal cells are reported to be the cells of origin of prostate cancer.[8,9]

The stromal to epithelial cell ratio is 5:1 in human and 1:1 in rodent prostates.[10] Despite this major histological difference between human and rodent prostates, both rodent and human prostate stroma are mainly composed

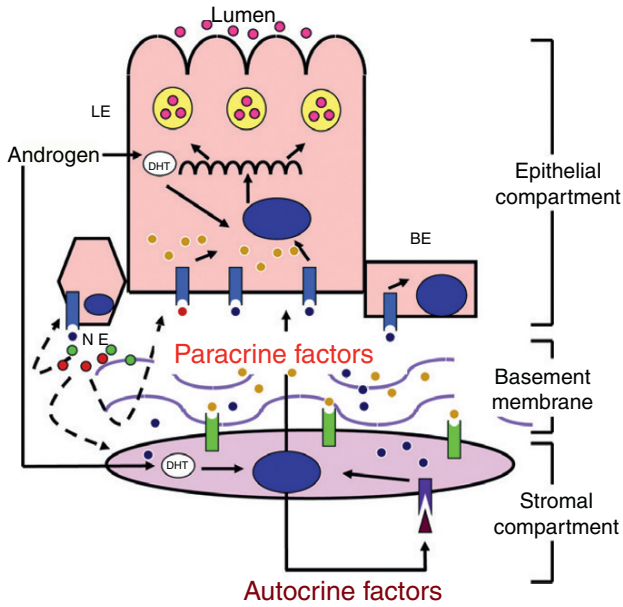

FIG. 1. Compartmentalization of the prostate. The prostate epithelium consists of secretory, basal, and neuroendocrine (N) epithelial (E) cells, separated from the stroma by the basement membrane. Testosterone, converted to dihydrotestosterone (DHT) within cells, controls direction-specific paracrine and autocrine factors in both the stroma and epithelium, which interact with their cognate receptors and mediate regulatory functions of the androgens. The growth factor signaling pathways also modulate the expression and transactivation activity of the androgen receptors. These regulatory communications between the stroma and epithelium control the growth, differentiation, and apoptosis of the prostate cells and are important for maintaining tissue homeostasis in the prostate and androgen-dependent nonmalignant tumors.

of specialized smooth muscle cell (SMC)-like and diverse other nonparenchymal cell types, including matrix-producing fibroblast-like cells, cells contributed by innervations, the immune system, and the circulatory system. The SMC-like cells, which express α-smooth muscle actin, intimately associated with and surround the epithelial cells. The fibroblast-like cells and other stromal cells are more dispersed within the stromal matrix. In rat prostate tumors, the reduction of SMC-like cells in respect to the number and degree of differentiation is found to be associated with the progression to malignancy.[11] Since loss of SMC-like cells and dominance of the undifferentiated fibroblast-like cells are found associating with tumor progression in rat prostate tumor models, it appears that the epithelial cells are essential for maintaining the properties of SMC-like cells as well as the overall cellular composition of the stroma.[11]

Although rodent prostates seldom develop spontaneous prostate tumors, Dr. W.F. Dunning of the University of Miami observed a spontaneous papillary adenocarcinoma of DP of a Copenhagen rat in 1961,[12] which was designated as the Dunning tumor. The tumor can be maintained in male syngeneic Copenhagen rats. After a series of passages in both intact and castrated hosts, several sublines of tumors had been developed, including both hormone-sensitive and -insensitive tumors.[13] The progression of the Dunning tumor model recapitulates what happens during human prostate tumor progression and has been widely used to understand how prostate tumor progress and become castration insensitive.[14] Yet, genetic manipulation in rats is not well established, which limits the development of new prostate tumor models in rats and hinders in-depth investigation of the onset, progression, metastasis, and relapse of prostate cancer at the molecular level.

Recent progress in mouse genetic engineering technology, including forced expression and gene ablation, has led to the generation of a series of genetically engineered mouse (GEM) models of human prostate cancer to mimic human prostate tumor initiation, progression, and metastasis. These models are generated based on potential etiological factors of human prostate cancer, such as inducing genetic instability or deregulating cell signaling, which cause dysregulation of proliferation, differentiation, apoptosis, homeostasis control, and other cellular activities. The major advantage of GEM models is that they provide a model system for evaluating the role of a single gene and its interaction with other genes or environmental factors in prostate cancer initiation, progression, and metastasis. These models can be used for testing new therapeutic or preventive agents in intervention and prevention of prostate tumor progression at various stages and for identifying molecular mechanisms by which these therapeutic and preventive agents exert their actions.

II. Technologies for Creating GEM Models

A. Overexpression

A wide spectrum of genes has been shown aberrantly expressed or mutated in prostate cancer cells, implying their roles in the onset, progression, metastasis, and relapse of prostate cancer. Therefore, forced expression of these genes in the prostate will provide *in vivo* assay systems to scrutinize their roles in prostate cancer. Generally, the mice that overexpress genes of interests in targeted tissues can be categorized into two types, transgenic mice and knockin mice. The conventional transgenic mice are the most common ones, which are referred to mice carrying an artificial gene or exogenous cDNA that is introduced into the mouse genome by microinjection into the pronucleus of

fertilized eggs. A typical transgene includes a promoter that target the expression to specific cell types, a short intronic sequence that is required to ensure the integrity of mature mRNAs, a cDNA that encodes the protein of interest, and polyadenosine addition sites. Normally, multiple copies of the transgene are integrated into the genome randomly in tandem repeat manner. Although expression of the transgene is driven by the promoter, the chromatin structure of the insertion site strongly affects the expression level. The impact of the chromosome structure, however, at least partly, can be overcome by inclusion of an insulator element in the transgene. An insulator is a DNA fragment that associates with strong DNase 1 hypersensitive sites and tends to separate chromatin domains with different degrees of condensation, thus, minimizes negative effect of chromosome structures.[15] The transgenic technology provides an easy and time-saving way to create overexpression models. A highly active and tissue-specific promoter is critical for targeting the expression to a specific cell type. Yet, the expression levels vary with each strain, and insertion of the transgene may impact or disrupt normal function of the inserted allele. Thus, precautions have to be taken in phenotype analyses.

The "knockin" technology is another way to create forced expression models in which the coding sequence of a protein of interest is inserted precisely into a desired genomic location by genetically engineering technology in mouse embryonic stem (ES) cells. The expression of the knockin coding sequence is controlled by the normal transcriptional machinery of the knockin allele. The advantage of knockin models is that the expression pattern and the outcome of disruption of the host allele are predictable. One common allele for ubiquitous expression without obvious side effects is the ROSA26 allele.[16] Yet, this is a time-consuming and tedious way to generate knockin mice, and sometimes, the expression level may not be high enough. Together with either the Cre/loxP recombination or other gene expression regulatory mechanisms, such as the tetracycline controlled gene expression system, more sophisticated expression systems have been used to express genes of interest in mice. Instead of being directly controlled by the promoter only as in conventional transgenes, expression of these conditional expressing transgenes is further regulated both temporally and spatially either by Cre/loxP mediated recombination or by tetracycline regulatory transcription factors.

1. Prostate-Specific Promoters

The rat probasin (PB) promoter and its derivatives, which include the minimal PB promoter,[17] the long 12 kb PB promoter,[18] and the composite ARR2PB promoter,[19] are the most commonly used promoters for targeting expression of genes of interest to the prostate epithelium. PB is an abundant protein that belongs to the lipocalin superfamily. PB is located in the nucleus of prostate and seminal vesicle epithelial cells, as well as in prostatic secretion

fluids. The LP has the highest expression level of PB, followed by the dorsal, anterior, VP, and seminal vesicles. The expression of PB reaches the maximum level when the mice become sexually mature; androgen ablation quickly decreases PB expression, indicating that the expression is regulated by androgens. Detailed characterization reveals that two distinct AR-binding sites, ARBS-1 and ARBS-2, are required for maximal androgen-induced gene expression.[20]

The minimal PB promoter includes 426 basepairs of the PB gene immediately upstream of the translational initiation sites and 28 basepairs of the 5′-uncoding region downstream of the transcription initiation site. It targets transgene expression specifically to epithelial cells in LP, DP, and VP at moderate levels and the AP and seminal vesicles at low levels.[17] The transcription activity of the minimal PB promoter can be detected as early as 2 weeks after birth. The expression reaches maximal level by 7 weeks, which is correspondent to the sexual maturation. The minimal PB promoter has been widely used for expressing transgene in the prostate epithelium. The modest level of transcription of the minimal PB promoter makes it suitable for expressing gene products with high impact even at low expression levels, such as viral oncogenes. However, it is unsuitable for expressing genes that need high expression levels to have impacts. A large PB (LPB) promoter fragment composed of 12 kb upstream sequence of the PB transcription initiation site is used to achieve high level expression in the prostate epithelium.[21] Similar to the minimal PB promoter, the expression activity of the LPB promoter is also androgen regulated. However, its bulky size makes it difficult to handle and reduces the efficiency of cloning and genomic integration.

A composite ARR2PB promoter has been made in which the inhibitory sequence between -426 and -287 in the PB promoter was removed and replaced with two copies of the AR response region of the PB promoter.[19] The composite promoter, although small in size, confers high androgen-dependent expression of transgenes in the prostate epithelium. Compared to previous PB promoter, ARR2PB driven expression is more consistent and at high levels. However, low level activity has been noted in some other tissues, suggesting that the specificity may have been compromised as a cost of high expression levels. *In vitro* experiments also show that the ARR2PB promoter gives basal expressions in PC-3, LNCaP, and DU145 prostate cancer cell lines and the expression is significantly induced by androgens, whereas in nonprostatic cell lines, the transcription activity is very low and not androgen responsive.

Prostate-specific antigen (PSA) is a kallikrein-like serine protease that is almost exclusively expressed in human prostate luminal epithelial cells, and its expression is androgen regulated. The 4 kb immediately upstream sequence of the PSA coding sequence, which includes the proximal promoter and a strong enhancer region, has been used to direct expression of transgenes in the

prostate epithelium. The promoter delivers transgene expression to the LP by 8 weeks after birth at an androgen-dependent manner. The expression declines after androgen deprivation and can be restored by androgen replenishment.[22,23] In addition to the PB and PSA, several other promoters have been used to target expression of transgenes to the prostate epithelium with various degrees of specificities, which include the C3(1) promoter,[24] the fetal G-globin promoter,[25] the gp91-phox promoter,[26] the cryptdin 2 promoter,[27] MMTV,[28] and the 3.8-kb fragment of the PSP94 promoter.[29] Currently, no prostate basal cell- or stromal cell-specific promoters have been reported. Although not prostate specific, the p63 promoter is an obvious candidate for the basal cells since p63 is specifically expressed in basal cells, but not in luminal epithelial and stromal cells of the prostate. The generally stromal-specific fibroblast-specific protein-1 (fsp1) promoter has been used to express Cre recombinase in stroma cells, which has been shown to effectively ablate target genes in prostate stroma.[30,31]

B. Gene Targeting

Loss of function of tumor suppressors, such as Pten and p53, etc., through mutation, deletion, or epigenetic modification, is also common in prostate cancer. Analyzing mutant mice deficient in genes of interest is important in determining the function of these genes in the onset, progression, metastasis, and relapse of prostate cancer. Gene ablation based on homologous recombination in mouse ES cells enables the production of mutant ES cells that carry one mutant allele of gene of interest, which are subsequently used to generate chimeric mutant mice via microinjection to blastocysts.[32] If the ES cells have germline integration, the heterozygous mutants can be generated via breeding. In some cases heterozygous mutant mice are sufficient, but, in most cases, homozygous mutant mice are needed to produce phenotypic changes. The convention method is to directly delete a segment of DNA via homologous recombination, which has been widely used to generate genome-wide knockout, also known as germline knockout. This conventional method has been used to investigate genes that contribute to the onset and progression of prostate cancer. However, if their disruption leads to embryonic lethality or severe abnormalities in mice, this approach does not permit evaluation of function of those genes in prostate cancer since prostate diseases only occur in adult animals. Furthermore, dissecting the role of genes specifically in the prostate is often complicated by systemic defects since germline knockout mice have the deletions in all cells and tissues. Abnormalities observed in the prostate may arise indirectly from developmental defects or functional defects in other organs.

The Cre/loxP- and the Flp/FRT (Flp recognition target)-mediated DNA recombination systems provide a more sophisticated technology for spatially and temporally specific gene ablation. Together with prostate specifically

expressed Cre or Flp recombinase, this technology greatly increases the power to functionally study the genes of interest in the prostate, especially those needed for embryonic development. The Cre/loxP system is derived from the bacteriophage P1 in which the Cre recombinase binds to two loxP sites and mediates recombination of the sequence flanked by two loxP sites.[33] If the two loxP sites are inserted in the genomic DNA in the same orientation, the recombination results in looping out the sequence between the two sites, leaving a single loxP site in the original DNA. If the two loxP sites are inserted in opposite directions, the recombination results in changing the direction of the intervening sequence. The loxP elements and a selection marker are normally inserted in the intronic sequences flanking the target coding sequence, which usually may not affect expression of the alleles. Therefore, together with transgenic mice that express the Cre recombinase in a tissue-specific manner, the Cre/loxP recombination is widely used for a temporally and/or spatially specific ablation of genes of interests. In addition to the Cre/loxP system, the Flp/FRT system has also been used for conditional gene ablations. The Flp recombinase is encoded by the yeast plasmid and catalyses a site-specific recombination reaction between two FRT sites.[34,35] Adding temporal regulation of Cre or Flp expression or activity further allows us to control gene ablation at a specific time point. The tetracycline-regulatable expression system has been increasingly used to control the Cre expression.[36] Fusion of the Cre recombinase and a mutated hormone-binding domain of the human estrogen receptor results in a chimeric protein, Cre-ER^{TM}. Since the Cre-ER^{TM} fusion protein can only be translocated to the nucleus and elicits its recombination activity in the presence of tamoxifen, the Cre-ER^{TM} mediated recombination is strictly under control of 4-hydroxytamoxifen.[37] The combination of tissue-specific expression and ligand-dependent Cre recombination will further provide precise timing and cell type-specific controls of gene disruptions.

1. Prostate-Specific Cre Lines

The first prostate epithelial-specific Cre line, PB-Cre, was generated by using the minimal PB promoter to target expression of the Cre in prostate epithelial cells.[38] Although the recombination efficiency is not high, the Cre driver is able to mediate the deletion of the floxed retinoblastoma (Rb) alleles in all lobes of the prostate, with the highest in VP and the lowest in AP. A second line of prostate-specific Cre transgenic line was generated subsequently, named PB-Cre4,[39] which carries the Cre cDNA under the control of the ARR2PB composite promoter. Expression of PB-Cre4 is postnatal and prostatic epithelium specific. Although the Cre recombination is detected in all lobes of the mouse prostate, the expression levels vary significantly in different lobes, being highest in the LP, followed by the VP, and then the DP and AP. Except for a few scattered areas in the gonads and the stroma of the seminal vesicle, no

other organs in adult PB-Cre4 mice demonstrate significant Cre expression. A third prostate epithelium-specific Cre driver, ARR2PBi-Cre, was generated with the similar strategy,[40] in which the expression of modified Cre recombinase[41] is driven by the ARR2PB composite promoter. An insulator element from the chicken globulin locus is inserted at the 3′-end of the transgene to minimize negative effects on transcription of the transgene imposed by chromosome structure. The ARR2PBi-Cre transgenic mouse specifically and uniformly expressed Cre recombinase in all lobes of the prostate, seminal vesicles, and ductus deferens. Compared with the other two prostate-specific Cre strains, the ARR2PBi-Cre strain exhibits higher and more uniform expression of Cre recombinase in the prostate, although it is also expressed in seminal vesicles and ductus deferens.[40]

The human PSA promoter is also used to deliver expressions of Cre[42] and Cre-ERT2 fusion proteins to the prostate epithelium.[43] The line PSA-CreD4 shows high, prostate-specific Cre activity in all lobes. PCR analysis shows that no other tested tissues exhibit Cre expression, which has been used to disrupt Pten alleles in the prostate epithelium.[42,44] The PSA-ERT2 is also specifically expressed in the prostate, with the highest expression in the DLP and lowest expression in the AP.

In addition, two knockin Cre lines, *Nkx3.1*^{Cre} and *Nkx3.1*$^{Cre\text{ERT2}}$, have been generated by knockin of the cDNA for Cre and Cre-ERT2 fusion proteins into the *Nkx3.1* allele, which are expressed in the precursors for prostate epithelial cells at embryonic day 17.0 when the prostate bud is formed. Although both Cre drivers are expressed in many organs, in the prostate, they are only expressed in the epithelium, which provide useful tools for studying genes of interests in prostate development[45,46] and in prostate stem cell research.[8]

III. Prostate Tumor Models Driven by SV40 T Antigens

The T/t antigens are the early genes of simian virus 40 (SV40), which include that large T and small t antigens. The large T effectively abrogates function of tumor suppressors, p53 and Rb, and causes genetic instability. In addition to suppressing p53 and Rb, the small t antigen also inhibits PP2A protein phosphatase activity, leading to overactivation of the MAP kinase pathway. Although the T/t antigens are not natural causes of human prostate cancer, they induce onset and progression of prostate tumors by disruption of genetic stability and unleashing cell signaling pathways that promote the tumor phenotype. In fact, several T/t antigen-driven mouse models resemble the development and progression of human prostate cancer in many aspects at an accelerated pace, which greatly facilitate mechanistic and preclinical studies

of prostate cancer. These models have been widely used for screening potential dietary factors and drugs for prevention and intervention of the onset, progression, and metastasis of prostate cancer.

A. The Autochthonous TRAMP Model

The autochthonous TRAMP (transgenic adenocarcinoma of the mouse prostate) model was developed based on the minimal PB promoter to direct expression of the T/t antigens to the mouse prostatic epithelium.[47] The T/t antigens are expressed in epithelial cells of VP and DP at moderate levels,[47] which can be detected at the age of 4 weeks when the mice are reaching sexual maturity. The combination of repression of tumor suppressors p53 and Rb and overactivation of the MAP kinase pathway causes prostate adenocarcinoma in a high incident rate. The TRAMP mice develop early prostatic intraepithelial neoplasia (PIN) by 6 weeks, and mild- to high-grade PIN by 12 weeks. At 24 weeks, approximately 100% of male mice have poorly differentiated and invasive adenocarcinomas. Metastases mainly to the periaortic lymph nodes and lungs can be detected as early as 12 weeks of age. Although the bone is the most common metastasis site of human prostate cancer, the TRAMP tumors, as other GEM prostate tumor models, rarely metastasize to bone.[48] The molecular mechanism underlying this difference is unknown. Therefore, to study the differences between human and mouse prostate tumors and to develop mouse prostate tumor models that have bone metastasis will provide hints for screening new therapeutic reagents for bone metastasis of human prostate cancer.

At early stages, the TRAMP tumors are androgen sensitive; castration of TRAMP mice at 12 weeks causes a regression of prostate tumors along with the normal prostate.[49,50] Similar to the human disease, although the castration at 12 weeks is curative in about 20% of TRAMP mice, the majority of the TRAMP tumors progress to poorly differentiated and androgen-insensitive cancers with frequent metastases by 24 weeks. As observed in human prostate cancer, TRAMP cells also undergo the epithelial-mesenchymal transition (EMT) marked by the loss of E-cadherin at later stages, as the primary tumors become poorly differentiated and metastasize. Therefore, the TRAMP model provides a consistent source of primary and metastatic tumors for histopathobiological and molecular analysis to further define molecular events involved in the onset, progression, metastasis, and relapse of prostate cancer.

TRAMP-C1, TRAMP-C2, and TRAMP-C3 are the immortalized epithelial cell lines derived from primary TRAMP tumors.[51] All three cell lines express cytokeratin, E-cadherin, and AR. TRAMP-C1 and TRAMP-C2, but not TRAMP-C3, cell lines are tumorigenic when grafted into syngeneic C57BL/6 hosts. The three cell lines represent various stages of cellular transformation and progression to androgen-independent metastatic disease, which have been widely used as an *in vitro* system parallel to the original mouse model for