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Advanced Models for Target Validation & Drug Discovery in Prostate Cancer

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1. Introduction

Prostate cancer (PrCa) is one of the most prevalent malignant diseases among men in Western countries. Despite good initial treatment response is observed in the vast majority of PrCa patients, tumor relapse is observed in about 7-10% of patients undergoing standard anti-hormonal therapies with anti-androgens and/or GnRH antagonists. There is currently no cure for **castration-resistant prostate cancer** (**CRPC**), and median survival for these patients is only about 18 months. The high mortality rate of advanced cases is closely associated with invasive carcinomas and systemic metastasis, most frequently to the bone; and CRPC is almost always coincident with overexpression/amplification of the **androgen receptor (AR)** gene. CRPCs fail to respond to all currently prescribed first line anti-hormones such as flutamide, nilutamide or casodex (bicalutamide). The second line anti-tumor treatment against CRPC, frequently taxanes like docetaxel, is administered to patients after the first or second relapse. Taxanes are often supported by zoledronic acid targeting bone metastases. These drugs and drug combinations are in most cases initially effective, but rarely curative. Although taxanes do show potent anti-tumor effects, advanced PrCa patients develop resistance and only gain several months of survival time. For decades, no major improvements have been made in the therapy of advanced PrCa. However, this situation has dramatically changed over the last few years with a number of novel, promising drug concepts in the pipeline, some of which have already entered the market. It is not expected that these drugs will be curative, and relapses and therapy failures are expected to develop in these cases which may remain fatal. Therefore, the demands will remain high to develop yet better drugs, and more faithful models that reliably mimic at least some of the key aspects of advanced prostate cancers. These models are required to recapitulate the mechanisms leading to therapeutic resistance & failure also for new compounds and drug combinations entering the clinics now. There will remain a need to mimic which mechanisms either in the tumor cells or in the tumor-associated microenvironment may have contributed to the resistance. This consistent high demand in improved models will go hand in hand with a better understanding of the pros and cons of the various models available. Continued development of improved model systems for PrCa and in particular CRPC could lead to an informed ranking according to the maximal throughput in drug screens that can be achieved, balanced against their true informative value and relevance for recapitulating clinical PrCa. A systematic side-by-side comparison of available models, e.g. cell lines, organotypic three-dimensional cultures and co-cultures, xenografts, tissue

recombination techniques and **genetically engineered mouse models** (**GEMMs**) is still missing. Furthermore, model development in PrCa is significantly lagging behind the advances in other fields, primarily breast cancer. Here, a considerably larger body of literature, methods, and better understanding of the pre-clinical models has been achieved, owing in part to innovative technologies such as 3D organotypic cultures and 3D tumor growth assays. Although prostate and breast cancer share many biological and histological properties and features, they remain fundamentally different diseases with different molecular pathways leading to transformation and progression. Modeling PrCa and in particular CRPC requires dedicated and specialized model development, coupled with a thorough characterization thereof.

In line with the relative lack of reliable PrCa models, and in contrast to other epithelial cancers, only a relative small number of molecular targets other than androgen receptor (AR) and AR-associated genes have been identified and validated in CRPCs (Feldman & Feldman, 2001). Even gene expression profiles of clinical prostate cancer samples were lagging behind studies related to e.g. breast cancers or gliomas, in particular characterization of advanced and metastatic lesions has been missing. Only recently, comprehensive large-scale transcriptome studies (> 250 clinical samples), combined with other levels of genetic analyses such as miRNA expression, next-generation sequencing, and analysis of mutations & copy number changes, have become available (Taylor et al., 2010). These studies will be invaluable for target identification and bioinformatic network analyses of PrCa's. Similarly, the identification of potent drugs that could block AR functions in CRPCs, such as novel synthetic anti-androgens, was mainly based on a relative small panel of largely reductionist models and cell lines (van Bokhoven et al., 2003a; 2003b). This situation has only slightly improved after the generation of a panel of new, more informative cell lines in the 1990's, and remains a key problem in PrCa research.

Additionally, many of the routine models for the pre-clinical phase of drug development are greatly insufficient, and fail to recapitulate key aspects of the molecular and biologic diversity of PrCa. For example, many PrCa cell lines actually lack expression of key molecular components such as AR or the critical ETS fusion factors. Furthermore, in particular in the early stages of pre-clinical research and cell-based screens, cell lines are routinely cultivated in monolayer on plastic. This does not support formation of **extracellular matrix (ECM)** nor relevant **cell-cell interactions** and epithelial **differentiation processes** to occur. Cell-cell-contacts formed by cells on plastic are of temporary nature, and fail to properly recapitulate differentiation and maturation programs intrinsic to epithelial cells. A key feature of both breast and PrCa is the capacity to undergo "acinar morphogenesis", i.e. the formation of functional glandular spheroids and tubular structures that connect such organoids. These are a key element of epithelial plasticity and contribute to cell motility and invasiveness (branching, spreading). No such features can be observed in monolayer cultures, depriving otherwise even potentially informative model systems of biological relevance. To overcome these shortcomings, organotypic cultures of breast and prostate cancer cell lines have been investigated since the early 1970's.

Cells can also be embedded in artificial or natural matrices or scaffolds. This provides an altogether different biology that has very little resemblance to the "floating" spheroids formed in non-adherent cultures, apart from the often equally rounded overall shape. Spheroid cultures or PrCa cells embedded in extracellular matrices have been systematically explored since 2001 (Bello-DeOcampo et al., 2001; Lang et al., 2001). This was again lagging behind the breast cancer field, in which such efforts were initiated in the 1990's (Streuli et al.,

1991; Weaver et al., 1995; Weaver et al., 1996). Later studies introduced a focus on tissue differentiation, morphogenesis and imaging (Debnath et al., 2003; Debnath & Brugge, 2005; Mailleux et al., 2008). The development of defined scaffolds/matrices is ongoing, although the most widely used material remains Matrigel. This is derived from a mouse sarcoma cell line that produces large amounts of laminin-rich ECM.

However, the most important shortcoming of currently available models for PrCa and CRPC is the lack of complexity that is recapitulated by mono-culture of a single cell type, usually the tumor cells. These do not act as singular entities and a carcinoma in fact represents a disturbed, but nevertheless complex tissue with its own regulation of tissue homeostasis. Tumor and stromal cells (in combination with ECM components such as laminins and collagens) are the main actors in tissue formation, followed by smooth muscle or myoepithelial cells and invading components of the immune system (Fig. 1). The development of co-culture systems of tumor cells with relevant tumor-associated or normal fibroblasts has not yet left the developmental phase. Despite a large number of different approaches, there is no consensus which of these models would be particularly informative and relevant. Most of the co-culture models may not be highly reproducible and remain only poorly standardized. Thus, more reliable models to study tumor-host interactions and the role of the stromal compartment in development and progression of PrCa are still much in demand. Also the role of tumor stroma in mouse xenografts is debated.

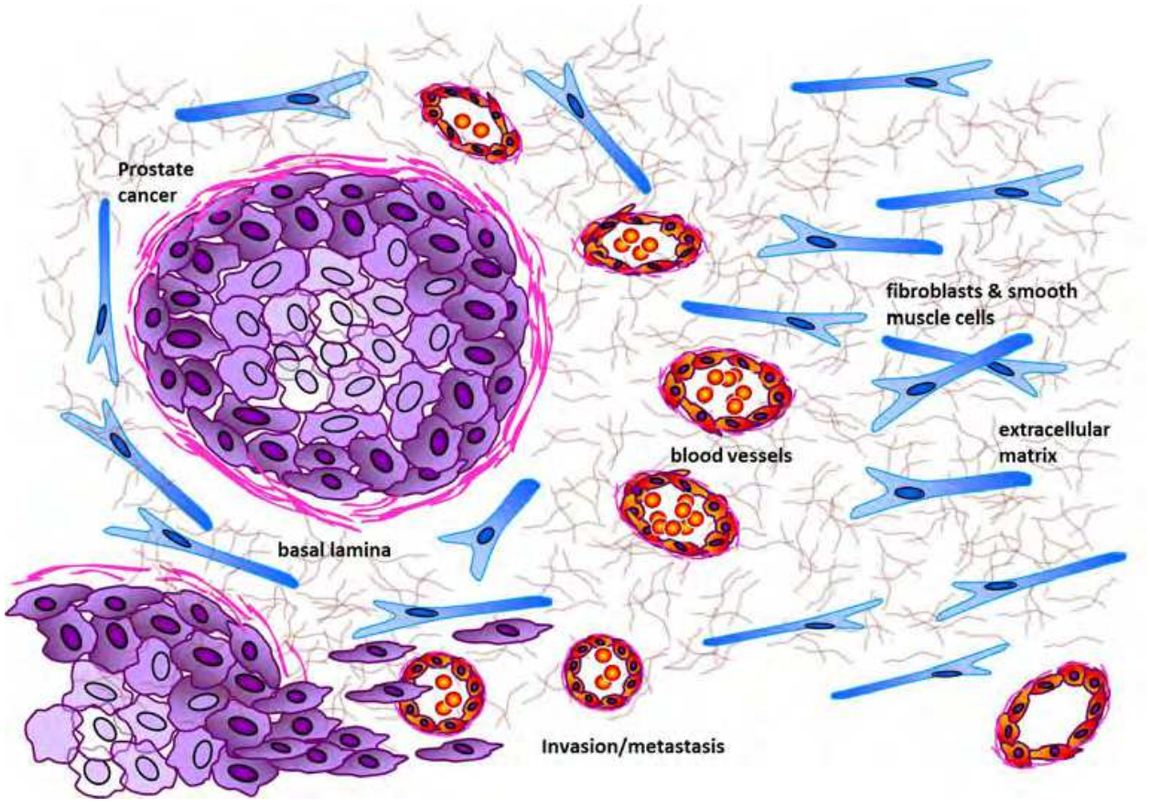

Fig. 1. Anatomy of prostate cancer. In low-grade tumors, cancer cells are still enclosed by intact basement membrane. The interstitial ECM is composed of fibrous proteins such as collagens, glycosaminoglycans and fibronectin. The cellular part is formed by fibroblasts, smooth muscle cells, myoepithelial cells, endothelial cells and invading actors of the immune system.

As a result, a lack of understanding of key molecular events in tumor progression to metastatic, invasive prostate cancers (CRPC) remains. Most models lack the required complexity, but also relevant high throughput capacities required for early stage pre-clinical research. Furthermore, most complex experimental systems are not cost effective. Many of the basic tools required for high content or **high-throughput cell-based screening (HTS)**, such as miniaturization, automation, and reliable readout systems are in a rudimentary state. Furthermore, those few model systems that allow higher throughput, may not be very representative for prostate cancer biology and differentiation. Such experimental systems are typically based on floating "prostaspheres" or organoids/microtissues; these, however, do not undergo significant differentiation processes. Only very few, matrix-embedded organotypic models are available for HTS. Apart from the lack of biological relevance of many experimental systems, it also remains generally difficult to transfer informative model systems across different laboratories. Together, these eminent insufficiencies may explain why preclinical studies are too often poorly predictive for the outcome of clinical trials, and why many of these trials eventually fail – typically in stage II or III. Generally, for a new compound to be synthesized and approved on the market it takes about 10 years or more, costing billions of dollars, and the number of FDA approved drug has steadily decreased during the last few years. At this point of the clinical drug discovery pipeline, a large sum of money has already been invested. Parts of it could have been saved, provided the target validation strategy had been more informative and pre-clinical models utilized had been more predictive. **Therefore, it is critical for future drug development to integrate multiple efforts, models and target validation strategies into a more comprehensive approach.** Only a broader spectrum of biologically relevant models allows thorough exploration of key mechanisms involved in therapy failure, and to focus on the major pathways involved in progression to CRPC. Furthermore, it will be mandatory to include the essential aspects of tumor-host cell interactions and tumor cell heterogeneity. This chapter will give an overview of the most relevant cell-based model systems currently available. We will mainly focus on in vitro cell culture models with an excursion into the wide field of orthotopic and subcutaneous xenografts. Only some selected examples for GEMMs that have been recently developed will be addressed here, in close connection to the cancer mechanisms and pathway they are modeling. Excellent reviews on mouse models of PrCa in general (Hensley & Kyprianou, 2011; Park et al., 2010) and GEMMS in particular (Jeet et al., 2010; Wang, 2011) have been published recently. According to this, we felt an overview of the status of ex vivo models may be timely, as this field is rapidly evolving and has not been widely reviewed. The most urgent unmet needs apply for the research related to fatal, under-treated CRPC. Our focus will therefore be mainly on the molecular pathways involved in progression to CRPC. Our aim is to discuss how advanced models may help to address improve target validation and drug discovery particularly in CRPC.

2. Target Validation: Modelling pathways and mechanisms in castration-resistant prostate cancer (CRPC)

2.1 Modeling AR modifications in CRPC

AR functions remain critical in essentially all CRPCs. AR is the target of genetic DNA amplifications leading to its overexpression (Koivisto et al., 1997; Visakorpi et al., 1995), as well as function-modifying point mutations. DNA amplifications targeting the AR locus result in overexpression of AR in up to 30% of the patients. Gain-of-function mutations of

AR (20-30% of patients) enable AR to bind a broad spectrum of steroidal and non-steroidal molecules as agonists (Koivisto et al., 1999; van de Wijngaart et al., 2010). Functionally relevant AR modifications include also changes in co-regulatory molecules such as nuclear co-activators and repressors. Additionally, alterations of signaling cascades that lead to activation of AR independent of ligand(s) have been described (reviewed in (Scher & Sawyers, 2005). In combination, these modifications are the most critical molecular mechanism that renders CRPCs independent of physiological levels of androgens. CRPCs thrive on significantly reduced levels of androgens or utilize alternative ligands that are more readily available. In some cases, AR mutations may render PrCa cells completely independent of external ligand supplies and the tumors develop an entirely independent, self-sufficient AR signaling axis – although AR still remains the main target. Ligand-independent and ligand-mediated functional activation of AR is reflected by phoshorylation, subsequent nuclear import (Jenster et al., 1993), and transcriptional activation of AR target genes such as **PSA (prostate-specific antigen)**. While PSA and other classic AR-controlled genes remain mostly driven by AR, the overall spectrum of AR-responsive genes is often greatly altered and expanded in advanced cancers (Wang et al., 2009a). This observation has only recently changed the basic understanding of CRPC biology.

Upon failure of primary therapy, anti-androgens such as casodex, flutamide or nilutamide fail to block AR and/or start promoting cell proliferation instead. These antagonists often convert to agonists (activators) of AR signaling, a poorly understood mechanistic complication (Dahut & Madan, 2010) - for which few experimental model systems exist. Many AR mutations described in patients confer gain-of-function properties. In the clinics, the conversion of anti-androgens and tumor relapse is reflected in a sudden steep increase of PSA levels. Nevertheless, PSA rise as such is only poorly indicative of patient survival and fails to predict response or failure of therapy. Instead of PSA, scintigraphy and PET imaging have turned out to be more reliable. However, the most promising method may be the detection of **circulating tumor cells (CTCs)** (Attard et al., 2009; Danila et al., 2010; Danila et al., 2011). CTCs typically contain the same cancer-relevant mutations as the primary tumor, e.g. amplified/mutated AR. These features can be utilized for further characterization and fine tuning of diagnostic tools (Attard et al., 2010; Zhang et al., 2010). However - the role of CTCs in forming distant metastases and relapse is only poorly understood. No good models are available that would dynamically mimic the systemic spread of PrCa cells. For example, xenografts rarely produce metastatic lesions to the bone, which represents the most frequent metastatic site in humans. Additionally, detection of small metastatic lesions by in vivo imaging is technically difficult (van Weerden et al., 2009), and may require removal of the primary tumor to allow monitoring the metastases. In vivo imaging may also suffer from extremely dynamic cell behavior in vivo: small lesions spontaneously disappear and reoccur at other locations, with few lesions able to successfully maintain themselves. It remains unclear how well these animal models represent the acute human problem of developing metastatic CRPC (Eaton et al., 2010). Major improvements in the use of light-emitting cell lines and more sensitive detection methods may help to overcome these difficulties in the future. Even more important would be the generation of reliable cell line models that effectively form metastatic lesions in mouse models – ideally utilizing mechanisms similar to human systemic spread.

Until very recently, little progress was made in the development of novel anti-androgens. The most exciting new entities, MDV3100, its derivative RD162 and TAK-700, were recently demonstrated as superior to casodex in castration-resistant LNCaP-AR xenograft models. Both MDV3100 and TAK-700 were also successful in clinical studies (Attard et al., 2011;

Chen et al., 2009; Massard & Fizazi, 2011; Tran et al., 2009). A phase I/II multi-center first-in-man trial of MDV3100 was recently completed (Scher et al., 2010), and a regulatory phase III trial with advanced PrCa patients previously failing taxane therapy has started in 2010. According to preliminary clinical data, MDV3100 does not quickly develop agonism to AR in CRPCs, and shows a more consistently and robust antagonist activity clearly superior to casodex. The still limited potential of these drugs is reflected in the possibility to generate MDV3100 resistant cell clones, e.g. *in vivo* by continued daily treatment of mice followed by serial passage of LNCaP/AR xenografts, or *in vitro* by using VCaP cells and long term drug exposure. In the majority of these resistant clones, AR expression is maintained. This is likely to happen in relapsed tumors from future clinical use. As expected, MDV3100-resistant CRPC cells remain dependent on continued AR signaling as demonstrated by siRNA knockdown: Not even the most advanced novel drugs are capable of breaking this addiction. In conclusion, novel drugs like Orteronel (TAK-700) and MDV3100 are unlikely to be curative, and will result in relapse and further progression with yet unknown frequency and time course. To explore the putative molecular mechanisms of MDV3100 or TAK-700 resistance in the future will require a very systematic approach and the use of a comprehensive panel of complementary models. There will also be a need to systematically address the impact of the tumor microenvironment and the stromal counterpart, using organotypic and co-culture models. These interactions are expected to considerable contribute to the development of late stage drug resistance, compared to treatment of primary tumors.

CRPC tumors may gain the capability to metabolically synthesize sufficient levels of androgens, which renders the tumors completely independent of endocrine hormone supplies (testis, adrenal gland). The drug **abiraterone acetate**, recently approved by the FDA for treatment of advanced CRPC, (Agarwal et al., 2010; de Bono et al., 2011; Molina & Belldegrun, 2011), blocks the formation of androgens by inhibiting CYP17A1 (17α-hydroxylase/17, 20 lyase). This metabolic enzyme is involved in the formation of DHEA and androstenedione. These intermediates are then further metabolized to testosterone (Attard et al., 2009a; Attard et al., 2009b). Like MVD3100 and TAK-700, abiraterone showed promising results in phase I-III clinical trials (Ryan et al., 2010; Shah & Ryan, 2010; Sharifi, 2010), but the response rate is equally incomplete and was approved by the FDA in April 2011. Abiraterone is also expected to result in resistances, with mechanisms that are very likely to involve the tumor microenvironment (stromal cells, endocrine factors and myoepithelial/smooth muscle cells). For example, the stromal cells may actually be responsible for developing resistance, subsequently providing significant levels of androgens to nearby tumor cells.

Nevertheless, recent success stories only illustrate that AR remains the fundamental target in PrCa, essentially throughout all stages of progression. Nevertheless, the novel drugs also demonstrate that it is possible to temporarily block AR or androgen functions even in CRPC. How well these new drugs will improve the efficacy of CRPC treatment, will be shown in the future. Understanding both the mechanisms of action & pathways leading to the expected resistance in tumor cells will require more than ever the use of a panel of advanced prostate cancer models. More detailed understanding of AR-related signaling pathways and an improved, contextual target validation will have the potential to significantly improve therapy and patient outcome. Ideally, this will include exploring the potency of combination therapies with older concepts, such as ketoconazole (Figg et al., 2010; Peehl et al., 2002; Ryan et al., 2010) or prednisone (Danila et al., 2010).

The insight that PrCa progression to CRPC is intricately associated with hyperactive androgen signaling was recently demonstrated by the generation of a mouse model based on an activating AR mutation (T877A), which was overexpressed in prostatic epithelial cells by targeted somatic mutagenesis. ARpe-T877A mice formed hypertrophies and eventually carcinomas (Takahashi et al., 2011). Tumor progression was greatly enhanced by overexpression of Wnt-5a that served as an activator. These findings suggest that mutant AR alone may already provide tumor-promoting properties which are further potentiated by additional genetic alterations. Such novel and exciting transgenic mouse models for PrCa may become very powerful tools in future pre-clinical trials (Jeet et al., 2010; Wang, 2011).

2.2 Modeling ETS fusion transcripts in PrCa & CRPC

AR may be the key player in PrCa progression but is certainly not acting in isolation. This was recently demonstrated by the discovery of TMPRSS2-ETS factor fusion genes that can be attributed to 40-60% of all PrCa (Tomlins et al., 2005; Tomlins et al., 2006; Tomlins et al., 2007; Tomlins et al., 2008). Other ETS fusion factors such as ETV1 and ETV4 (Hermans et al., 2008a; Hermans et al., 2008b) were soon following. The panel of ETS fusion rearrangements and driver genes/promoters is still growing, although the most frequent and relevant translocation is the TMPRSS2-ERG event. The occurrence of TMPRSS2-ETS factor fusion events is considered an important initiating event in PrCa tumor progression (Mosquera et al., 2008; Perner et al., 2007; Saramaki et al., 2008), but is not sufficient to fully transform benign prostate cells. This has been demonstrated by the fact that ETS fusion genes also act as tumor-initiating factors in transgenic mouse models (Brenner et al., 2011; Carver et al., 2009a; Carver et al., 2009b), but generally require cooperative oncogenic events such as haplo-insufficiency or complete loss of PTEN and activation of c-Myc (Sun et al., 2008) for progression to invasive carcinomas. Apart from the tumors, ETS fusion gene transcripts have also been detected in clinical pre-malignant lesions such as **HGPIN (high grade prostatic intraepithelial neoplasias)**. Surprisingly, TMPRSS2-ERG expression is frequently associated with a favorable prognosis (Hermans et al., 2009; Saramaki et al., 2008). Thus, ETS factor fusion genes events may represent cancer-initiating events, but might not critically contribute to tumor progression and CRPC. Also in clinical PrCa, ETS factors may need to cooperate with additional oncogenic events such as PI3Kinase pathway activation, loss of one allele of PTEN and AR amplification (King et al., 2009; Squire, 2009; Yu et al., 2010), which are considered key factors for tumor progression. However, this topic remains controversial: Duplication of the TMPRSS2-ERG fusion gene locus was associated with worsened prognosis and progression towards advanced CRPC (Attard et al., 2008; Attard et al., 2010). This also makes sense, as ETS fusion events are strictly androgen-responsive and require functional AR, which links ETS factors intimately to AR biology. Accordingly, TMPRSS2-ERG fusion gene expression is massively re-activated in CRPC tumors (Cai et al., 2009), concomitant with the over-expression of androgen-dependent genes like PSA. Thus, appropriate models for CRPC and the role of AR should not underestimate the contribution of ETS fusion genes, and their function cannot be clearly functionally separated from AR signaling. Only a small fraction of PrCa cell lines that contain actively transcribed ETS fusion transcripts have been described, such as the VCaP and DuCaP lines (Korenchuk et al., 2001). These cell lines harbor the characteristic AR amplification. Both VCaP and DuCaP, established from different metastatic lesions of the same patient, represent excellent models for both CRPC and ETS-factor positive PrCa. Nevertheless, these cell lines do not readily form metastatic lesions in xenograft mouse models (Havens et al., 2008; Loberg et al., 2007;

van Golen et al., 2008) nor do they belong to the most straightforward cell lines to grow in the laboratory – which somewhat limits their value. More advanced, aggressive models for CRPC based on VCaP cells have been generated using mouse xenografts (Loberg et al., 2006a; Loberg et al., 2006b). Apart from VCaP, only the NCI-H660 cell line contains another TMPRSS2-ERG fusion event (Mertz et al., 2007). But NCI-H660 is not a typical "luminal" PrCa cell line and lacks expression of AR, which renders this model rather irrelevant for many aspects of CRPC. This line was initially described as a small-cell lung carcinoma before it was reclassified as the metastasis of a prostate small-cell carcinoma. NCI-H660 may represent a model for the neuroendocrine differentiation phenotype, which is sometimes observed in PrCa. Furthermore, the classic castration-resistant, androgen-responsive LNCaP cell line contains a rearranged ETV1 fusion gene. However, ETV1 is not functionally expressed and LNCaP therefore not a very relevant model for ETS factor biology. Loss of ETV1 expression may indicate that this gene is not required for progression to CRPC. In summary, it is surprising that ETS fusion events appear to be under-represented in established PrCa cell lines. This observation may be related to particular difficulties to establish ETS-factor positive PrCa lines from clinical tumor material. The diagnosis of ETS factor fusion genes in PrCa have only now begun to affect clinical practice and diagnostics (Laxman et al., 2008; Tomlins et al., 2009), although detection of fusion events may soon become a routine technique (Hu et al., 2008; Jhavar et al., 2008; Mao et al., 2008). As of yet, the discovery of ETS fusion genes has also not resulted in many novel and useful therapeutic concepts (Björkman et al., 2008), although the first functional insights may yet have to be followed up and clinically translated (Gupta et al., 2010; Mohamed et al., 2011; Yu et al., 2010). A lack of appropriate models that faithfully mimic the biology of ETS fusions in the context of PrCa and CRPC may have contributed to the relative slow progress in this field.

2.3 Modeling molecular pathways beyond AR: PTEN, PI3 kinase and AKT

Alternative pathways for ligand-independent activation of AR are discussed as key mechanisms in at least a subset of CRPC. However, the kinases suggested to be involved, their clinical relevance, and the number of cases affected are still highly debated. Insights from large-scale tumor sequencing efforts such as the Sanger Institute (COSMIC database of somatic mutations in cancer; http://www.sanger.ac.uk/perl/genetics/CGP/cosmic) have identified candidate genes that are most frequently mutated in PrCa. According to this database (Status July 2011), p53 mutations are the most frequently found somatic alterations (19%), followed by PTEN (14%), and KRAS mutations (7%). Mutations in all three ras genes (HRAS, KRAS, NRAS) together account for about 13% of PrCa cases. Other frequent mutations, pointing essentially to the same key molecular pathways involved in prostate cancer progression, are EGFR (7%), beta-catenin (CTNNB1; 6%), Retinoblastoma (RB, 6%) and B-raf (4%). The frequencies for PTEN are significantly higher if genetic deletions (LOH) and rearrangements are also taken into account. Loss of one allele of PTEN is the most frequent genetic alteration in primary PrCa, while loss of both PTEN alleles is frequently observed in advanced CRPC. This genetic background information is critical to evaluate the biological relevance of models for PrCa and CRPC.

PTEN (phosphatase and tensin homolog deleted on chromosome 10) and the downstream **PI3 Kinase (PI3K)** and **AKT pathways** are closely linked to CRPC. PTEN loss and perturbation of these pathways have been implicated in early stage prostate carcinogenesis (Zong et al., 2009) as well as late stage CRPC (Verhagen et al., 2006; Vlietstra et al., 1998).

PTEN negatively regulates the activity of AKT and PI3K pathways. Loss of one or both alleles of PTEN increases the intracellular levels of the second messenger PIP3 and results in constitutive activation of AKT. PI3K promotes the activation of AKT at Thr^{308} via the kinase PDK1, followed by a second phosphorylation step by PDK2 at Ser^{473}. AKT then translocates into the nucleus and triggers many cell survival mechanisms, promotes cell cycle progression, and possibly invasion (reviewed in (Sarker et al., 2009)). Oncogenic, activating AKT mutations and gene amplifications have been described (Boormans et al., 2010). AKT's role as a primary survival factor may significantly contribute to the development of CRPC and therapy failure. Furthermore, PI3K and AKT are important pathways for the maintenance of PrCa stem cell populations (Dubrovska et al., 2009; Korkaya et al., 2009; Sarker et al., 2009) and stem cell survival. The PI3K pathway cooperates with other important proto-oncogene such as c-Myc (Clegg et al., 2011) in PrCa and model systems, and promotes cancer progression in both. Receptor tyrosine kinases (RTKs) like EGF Receptor, Her2/ERBB2, c-MET or IGF1R, as well as non-RTK's, also result in the activation of PI3K and AKT. Therefore, frequent observation of EGFR mutations in PrCa's functionally contributes to the same clinically relevant pathways, as do s Ras and B-raf mutations, which are downstream signaling cascades. PTEN loss, AKT and PI3K pathways have been functionally associated with ligand-independent AR activation mechanisms, but conclusive validation and precise functional details e.g. in clinical tumors are clearly missing. AKT may interact with and contribute to the ligand-independent phosphorylation of AR in CRPCs (Shen & Abate-Shen, 2007). Gain of function mutations in the PI3K pathway, primarily mutations of PIK3CA, are also the most frequent genetic mutation in breast cancers (Samuels et al., 2004), and occur sporadically in PrCa (< 1%).

Tissue-specific knock-out of a single allele of PTEN in mice promotes the formation of hyperplastic lesions but not carcinogenesis, (Korsten et al., 2009; Liao et al., 2010c). Haplo-insufficiency of PTEN strongly requires cooperation with additional tumorigenic events such as TMPRSS2-ERG fusion genes (Carver et al., 2009b; King et al., 2009; Squire, 2009), loss of NKX3.1 (Song et al., 2009), p53 mutations (Abou-Kheir et al., 2010; Couto et al., 2009), STAT3 (Blando et al., 2011), and AR overexpression. Loss of PTEN in the mouse prostate epithelium is generally insufficient to generate malignant lesions in transgenic mouse models (Couto et al., 2009), and typically results only in the formation of pre-malignant hyperplastic lesions. Interestingly, PTEN inactivation in mouse models without supporting additional events may primarily result in a specific form of senescence, which can be readily overcome by p53 knock-down (Alimonti et al., 2010). The resulting tumor cells show a dramatically increased stem- or progenitor cell and self-renewing potential (Dubrovska et al., 2009; Korkaya et al., 2009; Mulholland et al., 2009). It is also possible to isolate PTEN (-/+) mouse PrCa cell lines from primary tumors for molecular follow-up studies (Jiao et al., 2007; Liao et al., 2010c), and to further genetically modify these cells for generating androgen-independent CRPC lines. This includes the stromal compartment, which can also be extracted from transgenic mice and used for sophisticated tissue recombination and grafting experiments (Liao et al., 2010a; Liao et al., 2010b). Tissue recombination, e.g. combining mouse urogenital mesenchyme cells, or tumor-associated fibroblasts are a powerful model to explore tumor-host interactions and tumor microenvironment (Liao et al., 2010a). Cells from genetically engineered mouse models (GEMMs) can be further propagated in spheroid or 3D cultures, which may enhance the stem cell character of the resulting clones (Liao et al., 2010b).

The requirement for additional oncogenic events is also exemplified in mice in which loss of PTEN is combined with over-expression of the TMPRSS2-ERG fusion oncogene (Carver et al., 2009b) or loss of NKX3.1 (Song et al., 2009). NKX3.1 (-/-) PTEN (+/-) mice spontaneously develop androgen-independent lesions following castration (Abate-Shen et al., 2003; Gao et al., 2006; Kim et al., 2002; Ouyang et al., 2005), which renders this model particularly interesting for CRPC. Despite the availability of several mouse models generated by targeted knock-down of PTEN, only a few of these have resulted in castration-resistant tumors that represent human CRPC. In some instances, the physical or chemical castration of mice has been used to generate CRPCs (Banach-Petrosky et al., 2007; Shen & Abate-Shen, 2007). However, it is unclear how relevant this approach is as the endocrine production of androgens is different in mice and men. Furthermore, carcinomas will ideally form only after a very long time period, related to the natural aging process of the mouse (> 12 months). This represents a logistic restriction for the availability of such tumors/cell lines for larger scale experimentation.

Simultaneously, addressing the most AR-associated pathways (PI3K, AKT, mTOR) is incomplete without simultaneously incorporating upstream aspects of RTK signaling on these pathways and AR. The most relevant RTKs are most likely EGFR, ERBB2/Her2, IGF1R and the c-MET/HGF Receptors. In connection with AKT and PI3K activation, these RTK's and signaling modifiers like IGFBP2 (Mehrian-Shai et al., 2007) are important for the development and progression of PrCa; however their mutation spectrum and relevance in PrCa and CRPC is not very well established. Most critically, there are no PrCa animal models available yet that would systematically address these signaling mechanisms for generation of GEMMs. Oncogenic signaling and crosstalk through different RTK's, the variable functions and shifting roles of RTK's during therapy, tumor cell selection and the development of resistance, are likely to represent key mechanisms for target validation in anti-cancer therapy. Among the kinase receptors, the c-MET/HGF receptor pathway represents a particularly interesting target for CRPC and there is a need to recapitulate its molecular role by advanced model systems. Like EGFR, c-MET signaling appears to play a key role in many aspects of PrCa pathology (Szabo et al., 2011; Tu et al., 2010), particularly in regulating tumor cell motility, invasion and metastases (Pisters et al., 1995) as well as **epithelial-to-mesenchymal transition (EMT)**. Furthermore, c-Met/HGF signaling is possibly involved in the maintenance of cancer-initiating (stem-) cells and stem cell proliferation (Eaton et al., 2010; Pfeiffer et al., 2011).

2.4 Modeling tumor cell heterogeneity and mechanisms involved in cancer initiating or stem cells

An increasing amount of evidence implies a role for many additional mechanisms in progression to therapy-resistant cancers. This includes the overexpression of anti-apoptotic genes (Bcl-2 or Mcl-1, BIRC5/survivin), induction of the MDR (multi drug resistance) transmembrane pumps, activation of NF-κB, STAT2/3, and integrin-linked survival pathways (Weaver et al., 2002). These pathways may be critical for at least subsets of CRPCs. Cells that utilize additional survival mechanisms successfully may be strongly selected for under the conditions of anti-cancer treatment, and are likely to contribute to resistant cell clones. They are also likely to contribute to the generation of tumor cell heterogeneity before, during and after anti-cancer therapy. A spectrum of cellular factors like aneuploidy, differentiation and epigenetics are apparent in tumor tissues. Both genetic and non-genetic heterogeneities are likely to contribute to the clonal selection of resistant

tumor cells and/or tumor stem cell populations (Brock et al., 2009; Shackleton et al., 2009). Additional levels of heterogeneity are added by the cell populations from the tumor microenvironment (fibroblasts, myoepithelial cells), the immune system (monocytes, macrophages), and endothelial cells. During anti-cancer treatment, the hierarchical organization of tumors and their homeostatic regulation changes significantly. Together with the generation of genetically different clones, both aspects give rise to increasingly tumorigenic tumor cells & therapy resistance. The tumor context, tumor-host interactions and co-evolution with stromal components (Karnoub et al., 2007; Weinberg, 2008) are therefore critical aspects to understand clonal evolution and the nature of the resistant cells (Sawyers, 2007). However, there is a fundamental lack of reliable model systems to monitor dynamic changes in tumor & stromal heterogeneity (Marusyk & Polyak, 2010). The need for better systems to monitor epithelial plasticity is evident (van der Pluijm, 2011; Wang & Shen, 2011).

Alternatively, it has been suggested that residual disease and tumor relapse may be largely based on the long-term survival of **cancer-initiating cells (CICs)** or **cancer stem cells (CSCs)**. These rare and mitotically rather inactive cell populations have been suggested to persist during therapy, while the bulk tumor mass may yet be largely diminished. Stem cell populations may be intrinsically more resistant to chemotherapy (Diehn & Clarke, 2006; Diehn et al., 2009), and CICs may naturally acquire invasive properties, e.g. by undergoing EMT. CICs may therefore be largely identical to **metastasis-initiating cells (MICs)** (Mani et al., 2008; Polyak & Weinberg, 2009). In PrCa, the nature of CIC/CSCs and their association with EMT (Dunning et al., 2011) is debated. While it is widely accepted that PrCa contains a functional stem compartment, the molecular characteristics of CSCs remains unclear. It is not even established if these stem cells are of luminal or basal phenotype (Maitland et al., 2011). According to this uncertainty, there is a lack of appropriate and accepted models that address the biological relevance of CSC populations experimentally. The most relevant information may be derived from mouse models, while a role for stem cells in cell lines is even more debated. Some mouse models point to a rare luminal cell type (Wang et al., 2009b), while others describe molecular profiles more consistent with a basal and/or mesenchymal phenotype (Frith et al., 2010a; Giannoni et al., 2010; Kong et al., 2010), which is consistent with observations in the breast cancer field. Some models, such as tumor spheroid ("prostaspheres") cultures and non-adherent growth conditions, promote CSC properties and result in enhanced self-reproduction potential (Rybak et al., 2011; Watanabe & Takagi, 2008). This technique is now routinely used to enrich CSCs from mouse models (Liao et al., 2010a; Liao et al., 2010b) and human cell lines alike. Isolation of CSC populations from primary tumors (Guzman-Ramirez et al., 2009) and xenografts by serial passage of spheroids (Patrawala et al., 2006; Tang et al., 2007) or FACS reproducibly result in tumorigenic cancer-initiating cells. Their properties are similar to those generated by treatment with arsenic (Tokar et al., 2010a; Tokar et al., 2010b). The ultimate test for CSC characteristics is the inoculation of a limiting number of cells subcutaneously or into the mouse prostate, resulting in formation of tumors – although there are not as many studies compared e.g. to breast cancer CSCs (Al-Hajj et al., 2003; Dontu et al., 2005). It has been criticized that such assays may be an oversimplification and could simply select for the most tumorigenic cell populations. These may or may not coincide with CSC populations. The continuous debate indicates that also here, a lack of appropriate models (and biological understanding) limit the progress of drug discovery. Nude mouse models

(NOD/SCID) commonly used for inoculation experiments are far from fully representing the complexity of human malignancies. Generally, these models critically lack immune cells and lymphocyte-related cytokine/chemokine secretion. Furthermore, in xenografts, human cancer cells become rapidly associated with mouse fibroblasts. Even co-inoculation of cancer cells with human fibroblasts typically results in their rapid, effective replacement with mouse mesenchymal cells. Cell-cell interaction of human tumor cells with human stromal cells can therefore not be investigated. This poses a particular problem to the investigation of molecular pathways such as c-Met/HGF signaling (Tu et al., 2010; Yap & de Bono, 2010), in which the ligand is typically secreted by the stromal cells, while the receptor is expressed exclusively on the epithelial cancer cells. This represents a notorious problem for the validation of inhibitors and diagnostic tools alike (Knudsen & Vande Woude, 2008; Knudsen et al., 2009). Alternatively, it may be recommended to explore such pathways by mouse cells, accepting species-to-species differences. This will nevertheless assure that receptor-ligand interactions are fully functional. Tissue recombination approaches may also be very informative. The roles of non-genetic heterogeneity in clonal selection, and the various CIC concepts do not have to be mutually exclusive; both aspects may contribute to tumor cell resistance and failure of therapy.

3. *In vitro* models for prostate cancer

3.1 Two-dimensional monolayer culture: Cell lines and primary cells

Conventional 2D monolayer cell culture in combination with models like wound healing assays and transwell migration assays (Boyden chambers) have traditionally been the most straightforward and simplistic model systems for PrCa in vitro. This is due to the uncomplicated cell culture on plastic surfaces under controlled, and highly artificial environment. In vitro cell culture systems can be classified into two types: 1) cell lines, which have an unlimited proliferation capacity; 2) primary cell cultures directly established from human tissues. Cell lines are widely used in every aspect of cancer research and clearly represent the most common models. Cell lines have the big technical advantage of infinite reproducible quality (Rhim, 2000). Their growth properties and phenotypes are essentially dictated by the genetic background, which is largely defined by the genetic background of the original tumor. Therefore, different cell lines may show strong inconsistencies or contradictions that can be attributed to differences in the genetic wiring. These may nevertheless be representative of different stages and aspects of PrCa progression or differentiation (van Bokhoven et al., 2003b; Yu et al., 2009). The spectrum of mutations found in breast cancer cell lines was shown to be largely overlapping with primary cancers (Lin et al., 2007; Wood et al., 2006; Wood et al., 2007), although no such studies were performed for PrCa. If not single lines, at least panels of multiple PrCa lines may therefore be relevant for many experimental approaches. One problem with PrCa lines is the poor representation of the basal versus luminal phenotype that is characteristic for normal prostate versus PrCa tissues. Most PrCa cell lines are routinely cultured in media with 5 – 20% bovine calf serum, which strongly promotes the luminal phenotype. This is characterized by expression of the cytokeratins CK8, CK18, AR, and androgen-dependent genes. In contrast, most non-transformed, normal and immortalized prostate-epithelial lines are cultured in serum-free media. This strongly promotes the basal phenotype (Litvinov et al., 2006b; Uzgare et al., 2004), characterized by lack of AR expression and keratins CK5, CK6, CK7 and CK14. Luminal cell lines will stop proliferating in serum-free media, while

basal cell lines may adapt to serum-containing conditions, but still fail to undergo a luminal differentiation or start expressing AR. Therefore, it is questionable if basal-type primary cells and non-transformed prostate lines (RWPE-1, PWR-1E, or EP156T) are good models for clinical aspects of prostate cancers (Kogan et al., 2006; Tokar et al., 2005), as the luminal compartment is typically lost in malignant PrCa's (Litvinov et al., 2006b). Furthermore, the immortalization of primary prostate-epithelial cells with tumor-virus oncogenes (SV40 T-antigen, HPV-16 E6 and E7) typically results in rapid tumorigenic conversion. In contrast, the use of recombinant human telomerase has been demonstrated as far less compromising (Kogan et al., 2006; Kogan-Sakin et al., 2009). The resulting hTERT-immortalized cell lines retain much of their original differentiation potential, and do not accumulate additional genetic alterations.

Only an estimated 30 PrCa cell lines have been described, which were derived from clinical prostate cancer patients. However, only a small set of these cell lines has been widely used in cancer research. This also means that a large number of findings and scientific publications is based on a very small number of models. In particular, the three spontaneously established cell lines, PC-3, DU-145 and LNCaP, represent by far the most commonly used cell culture models (Sobel & Sadar, 2005a; Sobel & Sadar, 2005b), with close to 10.000 publications altogether. The first PrCa cell lines PC-3 (Kaighn et al., 1978; Kaighn et al., 1979) and DU-145 (Mickey et al., 1977; Stone et al., 1978) were established in 1978 and are still widely used. Both PC-3 and DU-145 have been cited in over 3000 publications. PC-3 cells were isolated from a human PrCa bone metastasis and have a very high metastatic potential (Kaighn et al., 1979), a property that has resulted in a large number of xenograft studies based on PC-3 cells inoculated typically into SCID nude mice. These xenografts are characterized by robust growth and rapid tumor formation. However, PC-3 cells are androgen-insensitive and lack expression of the AR protein. Loss of AR expression in PC-3 cells is likely related to epigenetic silencing of the AR locus. PC-3 cells may therefore represent a genuine subpopulation PrCa cells with naturally absent AR expression, characterized by very high cancer-initiating capacity possibly related to CSC. Interestingly, despite the lack of AR expression, PC-3 cells are capable of undergoing near complete acinar morphogenesis upon embedding in laminin-rich ECM (Matrigel). This argues for the substantial differentiation potential of PC-3 cells and in favor of their biological relevance (Härmä et al., 2010). Despite continuous ex-vivo culture on plastic dishes for over three decades, PC-3 cells have retained an amazing potential for epithelial maturation. Re-expression of AR in PC-3 cells can either suppress or slightly promote cell proliferation, depending on which promoter drives the expression of the AR protein (Altuwaijri et al., 2007; Litvinov et al., 2006a; Yuan et al., 1993). Additionally, PC-3 cells retain the co-activator profiles required for fully functional androgen signaling (Litvinov et al., 2006a). Nevertheless, such experimental strategies have to be taken carefully as they potentially contradict the genuine biological properties of a cell line.

Similar to PC-3, DU-145 cell were derived from a brain metastasis of human PrCa (Stone et al., 1978). Like PC-3, these cells are androgen-insensitive and lack expression of AR protein due to epigenetic silencing of the AR promoter by CpG island methylation that has shut off the expression (Chlenski et al., 2001; Yu et al., 2009). DU-145 cells show a similar, strong differentiation potential analogous to PC-3 cells when embedded in Matrigel. In both cases, a significant capacity for epithelial maturation has been retained for three decades of ex vivo culture. Stable transfection of functional human AR into DU-145 cells results in cells with reduced proliferation rate. When DU-145/AR cells are treated with testosterone,

proliferation rate and other properties are restored, implying that AR can still function as a regulator of proliferation of DU-145-AR cells (Scaccianoce et al., 2003). Taken together, both PC-3 and DU-145 cells represent interesting models, despite the lack of AR expression - although we lack a complete understanding of their biology and relevance for clinical PrCa.
The LNCaP cell line followed in 1980 (Horoszewicz et al., 1980; Horoszewicz et al., 1983) and has since resulted in over 5000 peer-reviewed publications alone. The LNCaP cell line was isolated from a lymph node metastasis, and contains a gain-of-function mutation commonly found in many clinical CRPCs (T877A). LNCaP cells are therefore a genuine and relevant model for CRPC (Yang et al., 2005). Down-regulation of AR in LNCaP cells by siRNA inhibits cell growth and increases the level of apoptosis (Compagno et al., 2007; Eder et al., 2000; Yang et al., 2005; Yang et al., 2005). This is suggesting that LNCaP are addicted to oncogenic variants of AR which act as a key survival factor; a characteristic or CRPC.
Newer cell line models have only been added to this small collection during the late 1990's. These cell lines are typically derived from xenograft models, such as the 22rV1 and CWR-r1 lines which are both derivatives of the CWR22 xenograft (Sramkoski et al., 1999). Similarly, the PC346 panel of cell lines was derived from a human xenograft and has been developed into a comprehensive series of derivative cell lines that mimic many aspects of progression to CRPC (Marques et al., 2006; Marques et al., 2010; Vlietstra et al., 1998) and resistance against anti-androgens. The PC-310 and PC-82 cell lines represent similar models (Jongsma et al., 2000). Another panel of more recently developed and functionally relevant PrCa cell lines (MDA-PCa-2a , MDA-PCa-2b) was established from bone metastases of a single patient in 1999 (Navone et al., 1997; Zhao et al., 1999). Similarly, the DuCaP (Lee et al., 2003; Lee et al., 2001) and VCaP (Korenchuk et al., 2001) cell lines were established from soft tissue and bone metastases, respectively, of the same CRPC patient in 1999. DuCaP and VCaP currently represent the only relevant models for ETS fusion factor positive PrCa. VCaP and DuCaP are also the only PrCa cell lines that harbor a wild type, but amplified and overexpressed AR gene, a hallmark of CRPC. Both VCaP and DuCaP cells have been successfully used for xenograft models, mimicking cancer-stromal cell associations and stem-cell biology (Cooper et al., 2003; Pfeiffer et al., 2011). A specialty of DuCaP cells is their dependency on co-existing mouse fibroblasts, which represent a carry-over from the growth of these cells in xenografts. However, the dependency of DuCaP cells on the mouse stromal counterpart can be broken, resulting in a morphologically very different phenotype.
Although additional PrCa cell lines are also available, detailed studies have revealed that many of these are in fact derivatives of the other cell lines or even non-prostatic lines (Sobel & Sadar, 2005a; Sobel & Sadar, 2005b; van Bokhoven et al., 2003b).
The few bona fide cell lines, almost all derived from metastases, do not span the complete range of PrCa phenotypes, and are not fully representative of primary PrCa. It is therefore not surprising that until very recently, the lack of a variety in PrCa cell lines has probably contributed to the failure of most small molecule inhibitors against CRPC in clinical trials. A few cell lines derived from benign prostate hyperplasia have also been established (Chu et al., 2009; Cunha et al., 2003). It cannot be excluded that long-term culture changes the biological properties of a cell line, a problem that is confounded by the mismatch-repair deficiency observed in many cell lines. Therefore, primary cultures of malignant prostatic cells and their normal epithelial counterparts would be in principle preferable (Peehl, 2005) to cell lines. As the interest in "personalized medicine" is rising, well defined primary material from clinically interesting cases would be expected to provide an excellent opportunity to follow up specific questions experimentally. Primary cell cultures from

clinical tissue specimens indeed offer a number of biological advantages, and are often considered to better reflect the characteristics of the original tissues. However, primary cultures are usually derived from primary adenocarcinomas, unlike PrCa cell lines that have been typically generated from metastases of CRPCs (Maroni et al., 2004; Peehl, 2005). These may therefore represent biologically different entities. Furthermore, primary cell cultures present significant technical difficulties because of the limited access, restricted lifespan and requirements for specific culture techniques. Thus, only very few studies have made use of series of primary prostate cancer cells for experimentation (Eaton et al., 2010; Guzman-Ramirez et al., 2009). At the same time, the intrinsic heterogeneity of primary tissues poses many challenges, and a relatively large number of clinical samples will have to be processed. The isolation of primary cell material from samples is notoriously difficult. In practical experience, isolation and even short-term cultivation will fail in the majority of cases. Unrestricted access to primary clinical material is critical to overcome these many limitations, but may also pose a logistic problem. The availability of dedicated and experienced personnel at the clinical site to systematically collect, process and store such material is mandatory. The clinical partners also have to properly address and document questions concerning tumor grading and staging, therapy response etc., patient relapse and survival. These represent invaluable data for bringing tumor cell behavior into the correct and clinically relevant context.

Normal human prostate epithelial cells and even primary PrCa cells often undergo approximately 10 - 30 population doublings, before they become senescent (Peehl & Feldman, 2004; Sandhu et al., 2000). This could point to the possibility that immortalization is not necessarily a prerequisite for PrCa growth in vivo, and may represent a good explanation for the difficulties in generating PrCa lines in the past. Despite these difficulties, analyses of tumor suppressor activity, gene expression and cytogenetics in primary cultures have unraveled many critical changes that are important for PrCa progression. Challenges that remain to be addressed before tapping the full capacity of primary cell culture as a reliable model system include standardized and greatly improved isolation methods, the unequivocal characterization of cancer- and normal epithelial stem cells, and the successful induction and maintenance of a differentiated androgen-responsive phenotype (Miki & Rhim, 2008; Peehl, 2005). The isolation of cancer- or normal associated fibroblasts is a different issue and usually considerably less complex.

3.2 Organotypic 3D culture models

In monolayer culture of PrCa lines, cells lose important and biologically very relevant properties like differentiation, cellular polarization, cell-cell communication and extra cellular matrix (ECM) contacts. Simultaneously, wound healing, inflammation, and hyper-proliferation are artificially promoted – which is the main reason why 2D monolayer cell culture only poorly represents tumor cell biology *in vivo*. Accordingly, the most effective small molecule inhibitors and chemotherapeutic drugs in 2D monolayer settings primarily target cell proliferation and mitosis. Other interesting drugs that may affect differentiation-related pathways or small cell populations instead of the rapidly promoting tumor bulk, are likely to go undetected in cell-based screens using monolayer cultures. Such drug candidates may be connected to cell-cell interaction, maturation, EMT and cancer stem cell turnover - aspects that that incompletely recapitulated in 2D monolayer culture. In the pre-clinical phase of drug discovery, this bias results in an unnecessary low predictive value of many in vitro experiments.

The culture of glandular epithelial cancer cells in a certain tumor microenvironment consisting of purified ECM, such as collagen, hydrogels or Matrigel, was established over two decades ago (Streuli et al., 1991; Weaver et al., 1995; Weaver et al., 1996), and initially based mainly on breast cancer models. Matrigel represents a reconstituted, laminin-rich basement membrane, which supports cell polarization, cell-cell- and cell-matrix interaction, and promotes re-expression of differentiation markers even in transformed lines (Bissell & Radisky, 2001; Streuli et al., 1995). Glandular epithelial cancer cells rapidly adapt to different microenvironments and can dynamically switch between alternative pathways that regulate proliferation, differentiation and survival. When embedded in an ECM like Matrigel or collagen, normal prostate epithelial cells differentiate into hollow polarized spheroids, characteristic for functional, glandular epithelial cells (Simian et al., 2001; Xue et al., 2001a; Xue et al., 2001b). They also develop a pronounced motility and rapidly re-populate the available space by branching and acinar morphogenesis. Similar to normal epithelial cells, PrCa cells can also move and invade the surrounding Matrigel, although their mode of migration is phenotypically different from the formation of collective, multicellular sheets or tubes of cells observed in normal cells (Fig. 2)(Friedl & Wolf, 2008). The phenotype of cancer invasion strongly depends not only on the cells, but also on the composition and density of the ECM, and can vary from amoeboid blebbing, mesenchymal fibroblast-like motility and multicellular streaming or chain migration (Friedl & Wolf, 2010). 3D models of tumor-cell invasion are thought to properly represent many aspects of cellular dynamics inside actual tumors, as the cells can utilize a comparable mode for sliding and squiggling through the mesh of the ECM (Fig. 3)(Wolf & Friedl, 2006; Wolf et al., 2007). Invasion in 3D is assisted by proteolytic processes and proteases (Friedl & Wolf, 2009; Wolf & Friedl, 2009) and soluble factors (Gaggioli et al., 2007). Furthermore, cell motility and invasion is also controlled by intrinsic physiological factors, such as re-organization of the cytoskeleton (Medjkane et al., 2009; Sanz-Moreno et al., 2008). The potential to undergo an EMT and to acquire mesenchymal migration modes is a critical aspect that is thought to contribute to PrCa invasion (Acevedo et al., 2007; Chu et al., 2009; Sequeira et al., 2008). Despite the need for 3D scaffolds, the most widely used cell culture models for tumor cell invasion are still comparably artificial assays such as transwell invasion assays (Boyden chambers), and wound healing/scratch wound migration assays. However, tumor cell migration in these models still occurs in essentially two dimensions. Any representative 3D invasion model that mimics motility inside the tumor microenvironment represents a significant improvement (Brekhman & Neufeld, 2009). Furthermore, 3D matrices more faithfully recapitulate genuine invasive pro-processes such as invadopodia formation (Harper et al., 2010; Wolf & Friedl, 2009), and embryonal, developmental processes that may be relevant also for cancers such as aspects that may be branching morphogenesis (Andrew & Ewald, 2010; Xue et al., 2001a; Xue et al., 2001b). The development of drug resistance also profits from appropriate 3D cell culture models. Drug resistance appears to be concomitant with increased cell motility and trans-differentiation features like EMT (Gupta et al., 2009; Kalluri & Weinberg, 2009; Reiman et al., 2010) also in PrCa (Armstrong et al., 2011; Giannoni et al., 2010; Kong et al., 2010). EMT has been intensely discussed as a key mechanism for future therapeutic options (Dunning et al., 2011) in PrCa. EMT and stem cell properties go together with metastatic potential (Chang et al., 2011; Li & Tang, 2011; Ling et al., 2011; Liu et al., 2011) and link EMT with the problematic of distant metastases. Also CTCs have been found to display properties of EMT, invasiveness and stem cell characteristics (Armstrong et al., 2011). To cover these morphologic and dynamic aspects properly, requires the combination of appropriate biologically relevant scaffolds that mimic

the tumor microenvironment, with appropriate cell lines (or primary cells) that show invasive properties. This could be counterbalanced by their variable differentiation potential, as epithelial maturation counteracts cell motility. Using such improved models, the screening for novel anti-cancer drugs can eventually enter a new phase. Researchers should increasingly utilize well characterized 3D organotypic model systems to explore the effects of drugs and targets in multicellular organoids. Appropriate models should also be cost effective and must provide sufficient throughput for high content screening.

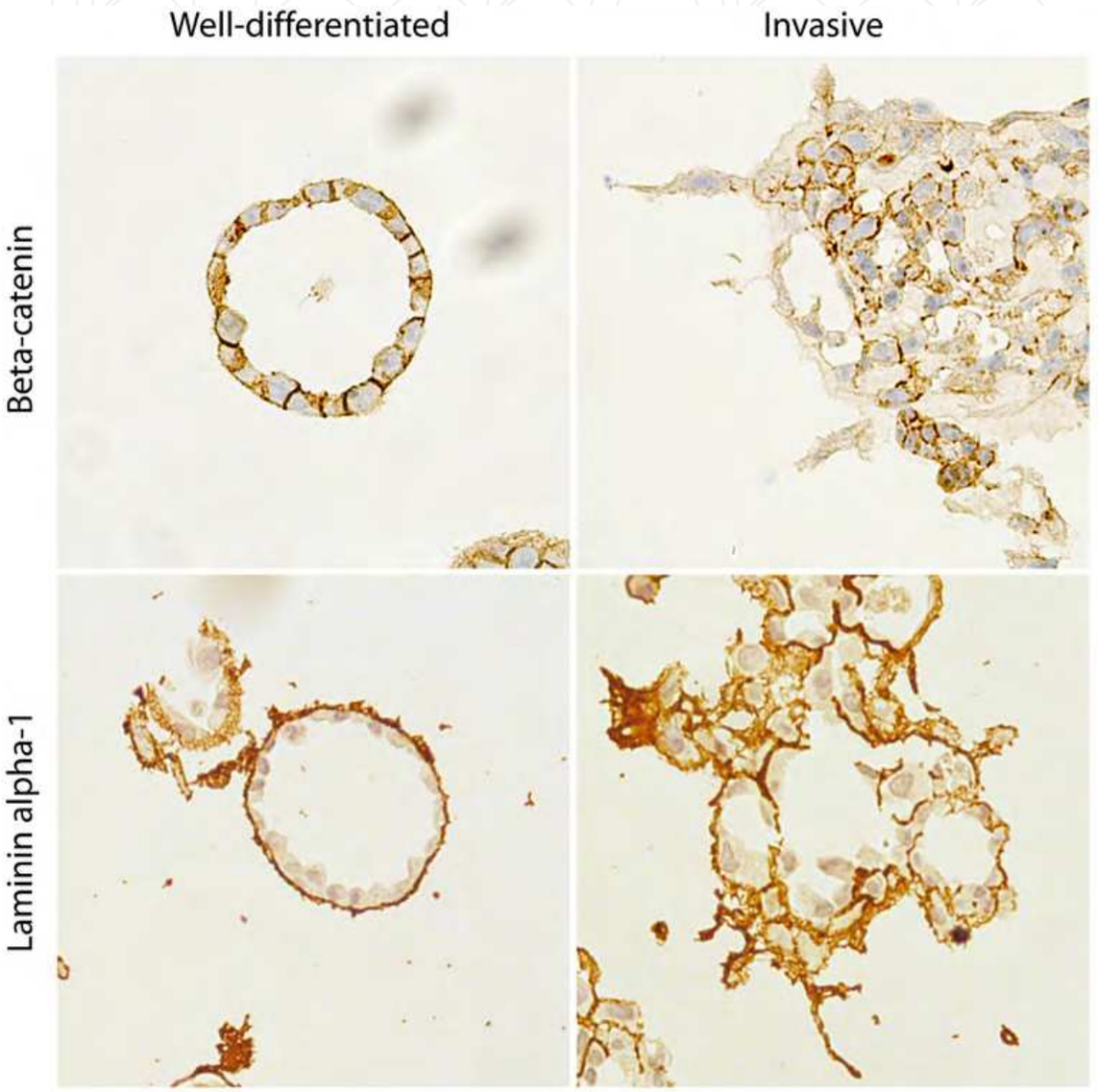

Fig. 2. Prostate adenocarcinoma cells in 3D laminin-rich microenvironment. Some PrCa cells bear the ability to differentiate into multicellular acinar structures with strong cell-cell and cell-ECM contacts (illustrated here with beta-catenin and laminin alpha-1 immunostainings). Invasive PrCa cells move by actively degrading the surrounding lrECM and tend to be less restricted by cellular contacts.

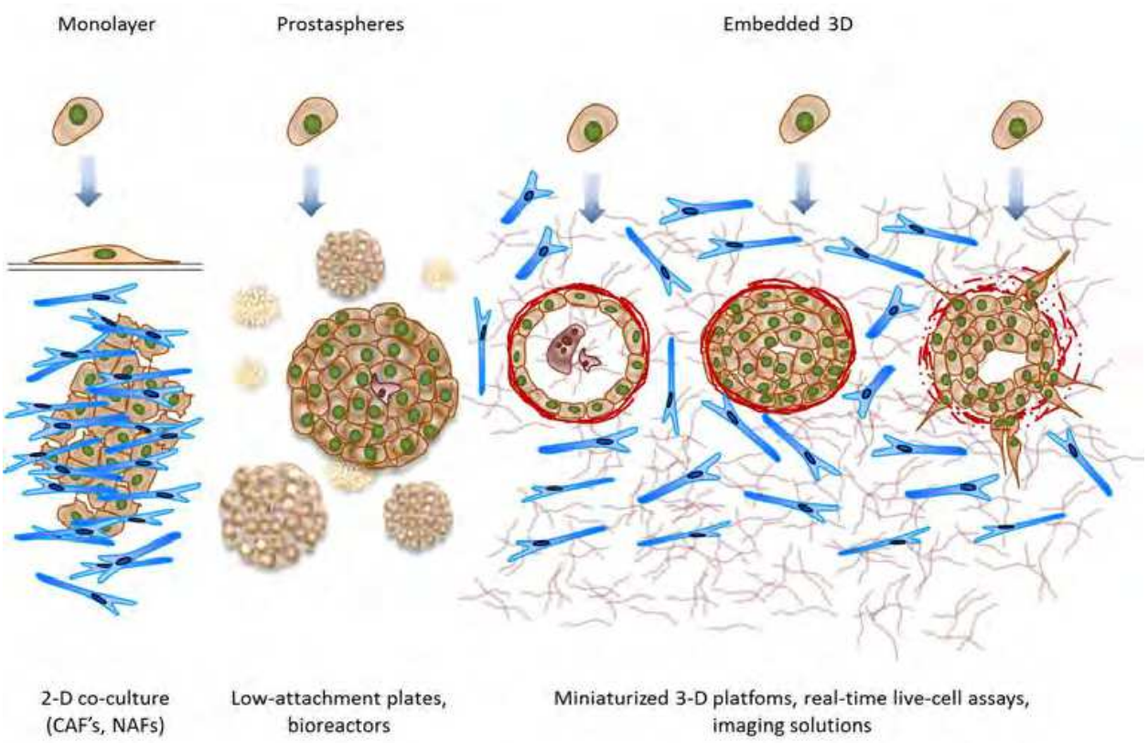

Fig. 3. An overview of three different in vitro models for PrCa: 2-D monolayer co-culture, low-attachment culture of prostaspheres, and organotypic 3-D culture.

In the pre-clinical phase of drug discovery, the question of drug response & resistance is routinely addressed by tumor xenograft models, mainly utilizing classic tumor cell lines and nude mouse models. While in vivo models may well overcome many of the shortcomings of 2D cell culture, they are time consuming, expensive, and require ethical considerations and permissions. Nude mouse models possess no immune system, and poorly mimic the critical aspects of inflammation, tumor microenvironment, human endocrine specialities and morphology, and often fail to mimic tumor metastasis. Xenograft models only allow remote sensing, followed by post-experimental histology. With few exceptions (in vivo imaging), animal experimentation gives no direct mechanistic insights into dynamic interactions between different cell types within a tumor at the cellular level. Monitoring inducible changes in real time is a key problem, potentially addressed in the future by tissue slices (Sonnenberg et al., 2008; van der Kuip et al., 2006). Until tissue slices may become firmly established, other models could represent a valid alternative. Therefore, 2D and xenograft experiments can be readily complemented by organotypic assays. These methods, which are still in development in PrCa, generate highly valuable functional data for drug discovery. Validated 3D models may also help to reduce animal experimentation and associated cost (3R strategy: "Replacement, Refinement and Reduction of Animals in Research"). Political pressure from the European Commission to reduce animal experimentation in Europe could further contribute to a wider acceptance of organotypic models in drug discovery. Advanced and well-characterized 3D models may give important clues for lead prioritization and allow significantly cutting the need for animal experiments, - for example if compounds successful identified in 2D screens are completely ineffective in subsequent 3D organotypic assays.

Generally, 3D cell aggregates (spheroids, prostaspheres, etc.) tend to show considerably higher drug resistance compared to 2D cultures. Such comparisons may result in a vastly different dose-response curve, ideally closer to the expected in vivo data. However, to firmly establish organotypic models and to validate their relevance in the pre-clinical phase of drug discovery, systematic side-by-side comparisons between in vivo and in vitro models would be required. These efforts are currently starting.

The biological relevance of functional screens conducted in 3D organotypic cultures strongly depends on information that can be concluded from associated assays. In routine cell-based screens, mostly indirect assays measuring overall cell viability and proliferation are used. Less frequent is the use of measurements based on biomarkers (e.g. Ki67; antibody stainings, immune fluorescence) or morphological parameters (imaging). These options are central for 3D organotypic cultures; the most important parameters for evaluating drug responses will be based on multicellular morphology. To functionally validate 3D organotypic models in cancer biology, high content microscopy based on morphological features will have to be combined with automated image analysis tools (Han et al., 2009; Han et al., 2010). This combination will allow real-time monitoring of dynamic changes in spheroid morphology as a readout. Automated image analysis of 3D cultures relies on measuring morphological parameters such as size, shape, differentiation, density, surface structures and invasive properties of spheroids. Consistent morphological changes in response to perturbants (small molecule inhibitors, siRNAs, stress conditions) can then be statistically evaluated and quantified. Apoptosis and cell proliferation can also be readily evaluated based on live-cell staining with reactive dyes. These processes are difficult to automate, and represent the key bottlenecks to overcome for larger scale screens. The use of GFP-tagged cell lines (combined with luciferase) represents a widely established tool that can be utilized in both in vivo and in vitro, organotypic settings. The same tagged cells can be used for in vivo and microscopic imaging, and could facilitate the much needed side-by-side comparisons of organotypic models with mouse models. Novel assays to monitor specific mechanistic changes will become important to quantify critical aspects of pharmacology and to evaluate acute drug responses. The field of live-cell assay development, including reactive dyes and reporter constructs, is a key aspect of HCS in the pharmaceutical industry but has yet to make the move into 3D models. There is a lack of informative assays for monitoring the activity of key pathways such as NF-κB, AKT, PI3K or Wnt in living cells. Such assays would also enable researchers to monitor cellular heterogeneity, e.g. in response to drug therapy. Single cell analysis, ideally based on live-cell assays, is already a powerful tool in many aspects of biology. This could also allow researchers to identify and monitor putative stem cell populations, and quantify their dynamics in anti-cancer treatments. Imaging is also critical to make sense of the dynamics of co-culture experimentation which will be discussed later.

3.3 Non-adherent 3D culture of prostaspheres and bioreactors

Early studies by Kinbara et al (Kinbara et al., 1996) demonstrated that prostate epithelial cells, isolated from different lobes of the adult rodent prostate, exhibited stem cell like features, including an enormous proliferative potential and the potential for re-programmed epithelial differentiation. Regenerative capacity, attributed to only a small population of pluripotent progenitor epithelial cells, is rapidly lost when the cells are placed in monolayer culture or embedded in Matrigel. However, “stemness” can be strongly promoted and

maintained by non-adherent cell culture, e.g. as spheroids. The first such approaches were described as "liquid overlay" technology, which prohibits successful cell attachment. Cells are then forced to adhere to themselves to overcome the lack of critical survival signals provided by cell and matrix adhesion, and to avoid anoikis or apoptosis. Low-attachment technologies have been introduced multiple times, e.g. by Poly-HEMA coated plastic plates. Placing prostate epithelial cells in non-adherent culture e.g. on a layer of hydrogel has a similar effect, resulting in the formation of spheroids or "prostaspheres" (Fig. 3)(Sauer et al., 1997; Sauer et al., 1998; Wartenberg et al., 1998). Such spheroids undergo a dynamic process of de-differentiation and reproducibly acquire properties of stem- and precursor cells (Patrawala et al., 2007; Pfeiffer & Schalken, 2010; Tang et al., 2007). Spheroid culture represents one of the oldest in vitro cell culture technologies and was pioneered already over 40 years ago by Sutherland et al (Sutherland et al., 1971; Sutherland et al., 1977).

Prostaspheres can be easily generated from most PrCa cell lines (Rajasekhar et al., 2011; Rybak et al., 2011), and serially passaged. Prostaspheres exhibit increased expression of putative stem cell markers, and represent 3D clusters of tumor cells derived from one or several cell clones that develop into multicellular globes of fairly large size. Spheroids often contain different subpopulations of cells that can be quiescent, hypoxic and necrotic and display a spatial geometry which provides a number of practical experimental advantages over adherent cell culture (Freyer & Sutherland, 1980; Kostarelos et al., 2004; Sutherland, 1988). To date, prostaspheres (and mammospheres, the equivalent in breast cancer cells) represent a widely used tool to study the processes of self-renewal, differentiation and cancer stem cell research (Lang et al., 2009). An intriguing recent application is the combination of spheroid culture to enrich for human prostate progenitor cells rat urogenital sinus mesenchymal cells. Inoculation of the formed chimeric prostate tissue under the renal capsule of nude mice (Hu et al., 2011) leads to tumor masses with human functionality, indicated by expression of PSA and hormone-dependent PrCa lesions. This represents an elegant system for the experimental recapitulation of carcinogenesis.

Another classic application for prostaspheres is within bioreactors (Ingram et al., 2010). These provide a low-turbulence environment which promotes the formation of very large and complex spheroids. A bioreactor is typically rotated or stirred, to provide a gentle mixing of fresh and spent nutrients without inducing excessive shear forces that may damage the structures. Bioreactors are also an ideal tool to generate co-culture spheroids, e.g. with stromal and epithelial/tumor cells (Yates et al., 2007a; Yates et al., 2007b), or to promote the self-renewal potential and stem cell characteristics (Frith et al., 2010b). Bioreactor research could be instrumental in helping scientists to prepare better models for cancerous tissues, and is expected to facilitate drug and peptide development. Improved characterization of these models and comparisons to other alternatives would be important also in this case.

3.4 Co-culture models for the investigation of tumor-stroma interactions

In a tumor, fibroblasts, smooth muscle cells, endothelial cells and leukocytes, interact physically or via the secretion of paracrine signalling molecules with tumor cells. These cell types make up the main components of the breast tumor microenvironment (Bissell & Radisky, 2001; Polyak et al., 2009; Radisky et al., 2002; Shipitsin et al., 2007). The situation in PrCa has also been explored in detail (Cunha et al., 2003; Cunha et al., 2004). Stromal cells secrete a variety of growth factors like FGF-2, FGF-7, and FGF-10 (Chambers et al., 2011), insulin-like growth factor (IGF), epidermal growth factor (EGF), and a panel of chemokines

(Kogan-Sakin et al., 2009). FGFs like FGF-10, which signals through the FGFR1 receptor, play a particularly critical role in regulating PrCa cell morphology and invasive properties (Abate-Shen & Shen, 2007; Chambers et al., 2011). Dysregulated FGF-10 expression has been observed in advanced PrCa and suggests the FGF-10/FGFR1 axis as a potential therapeutic target in treating both hormone-sensitive or CRPC (Memarzadeh et al., 2007). Exposure to paracrine growth factors like FGF-10 and FGF-7 may be critical for the initiation of oncogenic transformation (Fata et al., 2007). Additional secreted factors including chemokines like CCL2 act as key factors for PrCa invasion and bone metastasis (Li et al., 2009; Loberg et al., 2006a). These and others like IL-6 are thought to support differentiation and stimulate cancer cell growth (Culig et al., 1995; Malinowska et al., 2009). A tumor is clearly the product of intricate cross-talk between the epithelial and stromal cells; with the secretion of cytokines and chemokines as the common language. Identification of these environmental, paracrine cues which induce important changes in cell fate during development, has caused a fundamental re-evaluation of the process of tumorigenesis.

In vitro co-culture systems could also provide better models to address the interaction of epithelial AR functions in cell proliferation and metastasis (Fig. 3). For example, the immortalized human prostate stromal cell line WPMY-1 expresses functional AR and secretes paracrine growth factors (Webber et al., 1999), with an impact on the morphology and proliferation of the epithelial counterpart. Stromal AR expression promote epithelial cell invasion via paracrine secretion of growth factors, chemokines or cytokines. The stromal cells, via AR expression, can therefore modulate tumor cell proliferation and invasion (Tanner et al., 2011; Yu et al., 2011; Zhang et al., 2008). Their key effects are mediated e.g. by estrogen receptor signaling or the ERK kinase family, but also secreted high-molecular weight glycoproteins like endoglin (Romero et al., 2011). Expression of eccrine factors is mediated by TGF beta, another key factor and signaling pathway that massively affects the tumor-stroma interaction (Chambers et al., 2011; Pu et al., 2009). TGF beta dependent mechanisms play a key role in regulating paracrine stromal signals, and strongly affect epithelial cell adhesion via adhesion/cytoskeleton interactions. These and many other reports indicate a fundamental role for the stroma and stroma-derived secreted factors in maintaining adult prostate epithelial tissue morphology and integrity.

Simple, modular and reproducible 3D co-culture systems might allow researchers to further address the interdependence of tumor and stromal cells in straightforward settings. These systems would ideally allow the analysis of morphological features and epithelial differentiation/maturation. Optimal systems should also be standardized and miniaturized to facilitate the use of imaging and automated image analysis tools, and provide a higher experimental throughput. Such advanced, reproducible 3D co-culture models could then be utilized to systematically explore the importance of molecular signalling pathways AR, PI3K/AKT, and c-MET on heterogeneous co-culture and tissue formation. For example, stable stromal-tumor co-cultures are expected to be more resistant to anti-androgens or c-MET inhibitors than the isolated counterparts (Maeda et al., 2006; Tu et al., 2010), but this has not been properly investigated across multiple model systems. Which molecular and cell-cell interactions determine the response? Can resistance to anti-cancer therapeutics, e.g. androgen independence, be generated more readily in co-culture than by isolated mono-cultures, or even better in xenografts; and which molecular changes can be identified in resistant populations arising? What are the differences between human and mouse fibroblasts in terms of heterogeneity and response to anti-cancer therapeutics (Kiskowski et

al., 2011)? For such fundamental questions, the use of 3D co-culture settings could represent a critical "missing link" between reductionist cell culture models and more complex xenograft experiments or even GEMMs.

4. Conclusions

The heterogeneous nature of PrCa has made it difficult to understand the factors involved in the onset and progression of the disease. Advanced in vitro experimental systems should ideally try to recapitulate, as closely as possible, the 3D organization of tumors and mimic aspects of cellular heterogeneity and tumor-host cell interactions within the tumor microenvironment. Other important aspects are cell motility, the dynamics of clonal selection and tumor cell heterogeneity generated during chemotherapy. To provide a more comprehensive, biologically relevant context to investigate these processes experimentally, an ideal situation would be to combine in vitro models (2D, 3D and co-culture), transgenic mouse models (GEMMs), and xenografts into a bigger picture. A maximum of information can be generated by the systematic comparison of multiple models. The tissue architecture and heterogeneity formed by these various model systems may be vastly different, but the ultimate standard will be to relate these morphologies to the human clinical pathology and histology. Although the role of pathologists has not been featured prominently in this review, it remains one of the key aspects for cancer biology and the drug discovery process as a whole.

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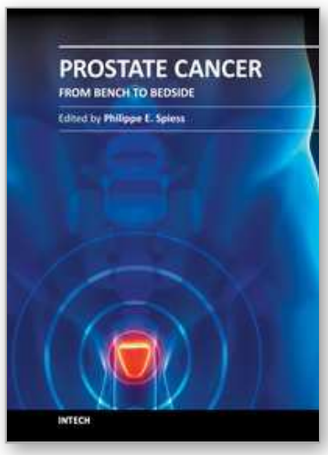

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The present textbook highlights many of the exciting discoveries made in the diagnosis and treatment of prostate cancer over the past decade. International thought leaders have contributed to this effort providing a comprehensive and state-of-the art review of the signaling pathways and genetic alterations essential in prostate cancer. This work provides an essential resource for healthcare professionals and scientists dedicated to this field. This textbook is dedicated to the efforts and advances made by our scientific community, realizing we have much to learn in striving to some day in the not too distant future cure this disease particularly among those with an aggressive tumor biology.

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