EXPERIMENTAL MODELS FOR THE DEVELOPMENT OF NEW MEDICAL TREATMENTS IN PROSTATE CANCER

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GENERAL OUTLINES

Although prostate cancer is a major cause of death and morbidity in western countries, one major obstacle in the progress of prostate cancer studies has been the limited number of relevant preclinical models. Even so, these models constitute the major preclinical tool required for the further development of new therapeutic strategies. Nevertheless, current limitations have hampered the transition of scientific findings from these models to human clinical trials. Each xenograft and genetically engineered mouse model possess inherent advantages and limitations regarding the mimicking of the natural history of the prostate cancer disease. Xenografts are the most commonly used models in drug development and are able to maintain several biological properties of the human tumours they are derived from, particularly when orthotopically transplanted. Osteoblastic bone metastases can also be modelled. Non-invasive methods of imaging have recently been developed and have improved the follow-up of the tumour growth and response to therapy in these models. In contrast, genetic models are very useful for target validation, but are less suitable for drug evaluation. Finally, some models are more relevant for the study of specific pathways as androgen receptor signalling, or bone metastases development, and others are useful in developing strategies targeting prostate cancer stem cells or mechanisms of resistance to chemotherapy or radiotherapy. As the perfect preclinical model does never exist, efforts are needed to better define questions and endpoints before choosing one particular animal preclinical model.

1. INTRODUCTION TO PRECLINICAL MODELS IN ONCOLOGY

a. Models in animals

The modern drug development technologies increase the number of candidate therapeutics, and subsequently increase the costs for preclinical and clinical testing. Thus the improvement of the ability of preclinical models to predict clinical efficacy can have a high impact by lowering the cost of drug development and by helping to prioritize compounds for clinical investigation. While the vast contribution of mouse models to advancing our understanding of cancer biology is indisputable, there have been ongoing concerns about the value of mouse models for predicting drug efficacy and usefulness in humans, and the predictive value of tumour xenograft models in cancer research is still a matter of debate.

Preclinical cancer models remain a highly valuable tool in the drug discovery process. As we continue to research and develop molecularly targeted therapies, xenografts are ideal systems for modelling biomarker responses in target tissue and toxicity in the host organism. Despite the debate around these models, there is an obvious need to develop better tumour models, and importantly, there is agreement that there is also a need to better define questions and endpoints for which animal models are used. Thus, selecting the right model is of crucial importance during the *in vivo* characterization of novel therapeutics.

The ideal prostate cancer model is one that reproduces the natural history of the disease, namely prostate tumourigenesis able to generate long-term spontaneous metastases in lymph node, lung and bone. This ideal model does not exist. It is therefore necessary to choose the most appropriate model for the signalling pathway targeted by the new drug to be tested.

b. Available preclinical models

1. Xenograft models

To pharmacologically evaluate molecularly targeted therapies, human cancer cell lines that exhibit a wide spectrum of prostate cancer characteristics can be implanted subcutaneously in immunodeficient mice. Such models offer good throughput by using cell lines that are readily replenished in tissue culture and by allowing assessment of tumour burden by simple calliper

measurements of the superficial tumours. Xenograft models offer a unique opportunity to evaluate the impact of drug candidates on tumour growth, pharmacodynamic target modulation and downstream signalling. In the case of pathway dependence, these models can be powerful tools to predict drug exposures required to completely inhibit pathway signalling. Importantly, combinations of specific targeted agents can also be used in human cancer cell line xenografts to inhibit multiple pathways that may be aberrantly regulated in the same tumour. However, these traditional subcutaneous xenograft models have many limitations which have led to the development of new models that are clinically more relevant. The major drawback of the subcutaneous xenograft models is the absence of an intact host immune system during cancer progression. Additionally, even if they may reproduce certain aspects of the tumour–host microenvironment, xenografts also lack several key features of the tumour environment and the absence of spontaneous metastases to clinically relevant sites. To address these concerns, many groups have developed orthotopic models implanting tumour cell lines or tumour fragments directly into the anatomical location of origin.

The orthotopic model is achieved by implanting tumour cells in mouse prostate glands, and although it's more difficult to do compared to subcutaneous xenografts, it has the advantage of frequently developing metastatic disease, thus mimicking disease observed in patients more closely. In many cases, orthotopic implantation of tumour cells has been found to greatly enhance metastasis compared to the same cells implanted subcutaneously. Nevertheless, the primary tumour often reaches maximum limits before metastases occur. Experimental modelling of metastasis can also be accomplished by directly inoculating tumour cells intravenously or intracardiactically. However, these approaches bypass the initial steps of metastasis, including local-regional invasion. Together, these results suggest that orthotopic transplant models may be better than subcutaneous transplant models, at least in reproducing certain aspects of the tumour–host interactions. Studying metastatic disease is possible in orthotopic xenografts, but it remains uncertain whether these models are more suitable than subcutaneous xenografts in predicting therapeutic benefit.

Non-invasive methods, including small animal imaging, have been developed recently for quantifying tumour burden for most disease sites. One important imaging modality, bioluminescence imaging (BLI), relies on the detection of light produced from cells tagged with luciferase and has improved the relevance of preclinical cancer models. The preclinical animal models can now be evaluated with a combination of imaging approaches that allow a rapid evaluation of tumour location and overall tumour burden, including metastases. This provides a number of benefits in the context of experimental therapeutic evaluation. As orthotopic xenograft models have been shown to provide a more faithful reproduction of human disease biology, the bioluminescent imaging constitutes an essential tool to non-invasively monitor tumour burden and response to therapy. Finally, since they are of human origin, orthotopic xenografts may be more likely to recapitulate molecular events involved in human prostate tumourigenesis than other experimental models.

- Direct implantation of tumour material into mice

Prostate cancer xenografts can be established in immune deficient mice by the subcutaneous implantation of fresh human prostate cancer explants surgically removed from patients with locally advanced or metastatic prostate cancer. The tissue from primary prostate tumours as well as from metastatic sites (lymph node, lung, bone and other organ metastases) is implanted into any subcutaneous site. Once established, the xenograft tumours grow and can be serially transplanted into other animals. Two important characteristics are required to use a prostate cancer heterotransplant as an experimental model. First, the resemblance between the serially heterotransplanted tumour in the host mouse and the initial patient specimen must be determined. Second, the fidelity with which the heterotransplant model reproduces the clinical outcome observed in patients must be characterized. This defines the predictive value of the

heterotransplant model which ultimately has to predict how human beings will respond to newly developed therapies (1).

The established heterotransplanted prostate tumours retained the biological properties of the original tumour, such as morphology, the degree of differentiation, pathology, secretory activity, and the expression of tumour markers. Human prostate tumour heterotransplants have considerable experimental advantages over cell culture following xenotransplantation because the human tumour architecture is preserved, allowing the stromal-epithelial cell crosstalk. On the other hand, the human stroma and vasculature are substituted as serial passaging progresses. The drawback of these models is the need for continued transplantation in animals, these models having been shown to be difficult to grow in *in vitro* culture. Additionally, because of the high transplantation failure rate, experiments involving serial passages *in vivo* require a large number of animals, which is expensive and laborious. Serial heterotransplantation results in androgen-dependent and androgen-independent heterotransplants, and accurately reproduces the clinical transition from androgen-dependent to androgen-independent disease. In this context, the heterotransplants have been used to evaluate new androgen deprivation agents (2-3).

- Tissue reconstitution models

In order to avoid some of the drawbacks of the models described above, an original *in vivo* model has been developed by several groups, consisting in the tissue slice graft of solid cancer, termed “tumourgrafts”, implanted under the renal capsule of immunocompromised mice (4-6). Recently, Zhao et al (7) have improved the method using precision-cut tissue slices to establish the prostate cancer models in one month that were maintained up to three months with a high engraftment rate. All the stages of the disease from premalignant lesions to well-differentiated cancer can be modelled, and prostate tissues responded to androgen ablation. It has been used to investigate the responses of prostate tissue to androgen manipulations. Thus it could be used to assess the antitumour activity of new drugs.

The tissue slice grafts implanted under the renal capsule retain the major histopathological and immunohistochemical characteristics of the original tissues and preserve an intact tissue microenvironment, thus providing the opportunity to address questions such as the role of androgen signalling in primary prostate cancers in a realistic *in vivo* setting. Potential applications of the tumourgrafts include preclinical testing of patients’ tumour responses to various chemotherapeutic regimens, evaluation of novel therapeutic agents and diagnostic methods, analysis of tumour progression at cellular and molecular levels, and the identification of new therapeutic targets. As for heterotransplanted prostate cancer models, a limitation is the tedious work of creating these explant models; human tumours must be freshly implanted. Furthermore, it appears imperative to utilize tumour specimens that do not just contain tumour cells to preserve the tissue environment influence of tumour growth. Importantly, these models are restricted to the study of localized prostate cancer and are not suitable for studying bone metastatic prostate cancer.

- Genetically engineered models

In cancer xenograft models, the study of human material grafted into the mouse has certain limitations, including a compromised immune system, differences in species specificity of proteins and an inability to capture early events in tumourigenesis. To overcome these limitations, the use of genetically modified animals provides another possibility for modelling prostate cancer and many genetically engineered models (GEM) in which tumour formation is driven by clinically relevant oncogenes, or loss of tumour suppressors, has been developed in the recent years. The ability to reproduce human disease in a mouse model has also contributed to discovering and assessing possible therapies. Although genetic differences between mice and humans exist, many molecular pathways involved in human tumourigenesis are conserved in mice and may serve as a proof of principle for the role of a given gene or pathway in oncogenesis. Such models have contributed

significantly to our understanding of the molecular and pathological aspects of prostate cancer initiation and progression. The initial and best characterized transgenic mouse models, TRAMP and LADY, generated over 10 years ago, were developed by inserting the SV40 viral genes upon the control of the prostate-specific rat probasin promoter. As the relevance of viral proteins in the development and progression of human prostate cancer is still unclear, newly generated transgenic models have been produced, involving overexpression or knockdown of gene generally suspected of being involved in oncogenesis. Because several recent reviews report a detailed description of available transgenic mouse models in prostate cancer, they will be not presented here (8-10). Some of them that mimic the physiopathological and molecular features of human malignancy exist now. For example, the prostate-specific deletion of the murine PTEN gene leads to the development of PIN within six weeks and invasive androgen resistant adenocarcinoma within nine weeks (11). Another transgenic model has been generated in which the dual inactivation of the tumour suppressor gene Nkx3.1 and PTEN results in an invasive carcinoma with androgen independence and lymph node metastasis (12-13). However this model is only obtained within a year. Invasive carcinomas were also obtained by the synergistic inactivation of p53 and Rb in mice presenting highly metastatic tumours that are resistant to androgen ablation and share several molecular features seen in advanced stage human prostate cancer (14).

The greatest advantage in studying tumourigenesis in the immunocompetent host is the ability to evaluate the role of the tumour microenvironment on the initiation, maintenance, and progression of the disease. Together with freshly transplanted human material, allografts of murine tumours from GEMs have resulted in the greater heterogeneity of tumour models that more closely mimic tumour-stromal architecture observed in the clinic. Depending on the homology between mouse and human of the pathway being studied, questioning tumour–stromal interactions in xenografts has been possible. Furthermore, these models enable the detailed study of the early stages of prostate cancer but are less accurate for studying the late stages of the metastatic disease. In addition, the major limitations of these models are the long latency of tumour progression and the limited metastasis in the single-gene mutation models. These models are also generally very expensive, and despite their unlimited potential, none truly encompasses the whole spectrum of prostate cancer.

2. EXPERIMENTAL MODELS TO TARGET ANDROGEN RECEPTOR AND AR PATHWAYS

The androgen receptor (AR) is the key molecule in both normal prostate development and prostate cancer. AR is a transcription factor belonging to the nuclear receptor family that activates the transcription of androgen-regulated genes such as the PSA gene. Upon binding of androgens, such as 5 α -dihydrotestosterone (DHT), AR dimerises, then is translocated into the cell's nucleus, and modulates the transcription of the target-gene after binding on the androgen-regulated element located in the promoter region of these genes.

Since the first observation by Huggins et al. in 1941 (15), androgen deprivation therapy (ADT) has become the major therapeutic option for advanced prostate cancer. Although initially effective in blocking tumour growth, ADT eventually fails, leading to a state of castration-resistant prostate cancer (CRPC). The AR paradox is that androgen independent tumour growth occurs even though the AR continues to be expressed in most cells of the CRPC. A recent study demonstrating that increased expression of the AR is the most common event associated with castration-resistant growth confirms the first observations (16). The detailed mechanisms involved in the conversion from androgen dependence (AD) to androgen independence in prostate cancer remain unclear. Subsequent castration-resistant growth of prostate cancer has been attributed to a variety of mechanisms that include AR mutation, with AR acquiring promiscuous binding to other steroids; imbalance of AR co-regulators, causing abnormal alteration of AR transactivation; alteration of selective androgen/AR signal transduction pathways through non-androgen induction; activation by receptor tyrosine kinases from growth factors, loss of cell cycle regulators or changes in the surrounding microenvironment and stromal cells (17). Moreover, recent studies of cell lines and prostate cancers have identified several alternative splice forms of the AR which arise after

castration and play a role in the progression of prostate cancer (18) (Table I). These AR variants have somewhat different structures, although each variant lacks portions of the ligand-binding domain (LBD), a feature predicted to produce a constitutively active receptor or dominant negative receptor. All the biologically active AR variants require full-length AR to activate endogenous androgen target genes and confer castration-resistant growth (39). A recently discovered AR^{V567ES} variant is constitutively active and able to increase expression of full-length AR in the absence of ligand (40). These findings suggest that AR variants could emerge as a primary cause of resistance to castration even though how they are generated is not yet known. The fact that the full-length AR is required for the AR variant function validates continuing efforts to develop even better anti-androgens targeting the AR ligand-binding domain.

- **Models to target AR signalling**

Taking into account the physiopathological functions of AR in prostate cancer, it seems crucial to choose the best appropriate model to develop new AR-targeting drugs. Data obtained solely from *in vitro* studies of human prostate cancer cell lines might not reliably predict the *in vivo* AR functions in prostate cancer progression and metastasis, in particular because several current available human prostate cancer cell lines were generated from long-term culture in the absence of androgen. That points up the importance of conducting *in vivo* animal studies of prostate tumours to delineate the pathologic functions of AR in prostate cancer progression and metastasis. However, the availability of a model that can be used both *in vitro* and in animals was clearly an advantage for studying AR-targeting drugs. It should be noted that some preclinical models derived from an original cell line could have been extensively manipulated, thus potentially introducing inaccurate genetic modifications. The most widely used human prostate cancer models that enable *in vivo* preclinical studies as xenografts to target AR signalling are shown in Table I. Note that many of them were propagated into xenografts and were not possible *in vitro*. As AR and AR variants are of central importance in the castration resistance progression, the appropriate models are still quite limited and the AR variant expression have not yet been identified in all of these models.

By analyzing a range of prostate models, the Charles Sawyers group has identified several structurally different AR variants with divergent biologic activity. All of those variants required full-length AR through dimerisation to activate endogenous AR target genes and confer castration-resistant growth (39). The new antiandrogen MDV3100 acting on the AR ligand-binding domain was shown to be active on CRPC patients in clinic (41-42). This compound abolishes the growth of LNCaP tumours expressing the AR-V7 variant, suggesting that MDV3100 could reverse the growth advantage of AR variants even if they were devoid in the ligand-binding domain and that AR variants require wild-type AR to confer gain of function through heterodimer formation (39). These results thus validate efforts to develop even better antiandrogens targeting the AR ligand-binding domain.

3. EXPERIMENTAL MODELS TO TARGET BONE METASTASES

As CRPC progresses, approximately 90% of the patients will develop bone metastases, and 20% will develop soft tissue metastases, most commonly in the lung, liver or lymph nodes. Currently, prostate cancer with bone metastases is not considered curable. The median survival of patients with bone metastases is approximately 20 months and is increasing with chemotherapy and other novel agents (43). Management of the quality of life in this patient group is therefore especially important and is likely to improve with the emergence of new bone-specific therapies. In patients with CRPC, bone metastases cause skeletal-related events that are responsible for substantial morbidity, and assessing bone health is an important aspect of clinical monitoring. PSA is the most widely used and best-characterized biomarker in CRPC; however, it does not provide accurate information regarding the extent of bone metastasis. In prostate cancer, bone metastases are predominantly osteoblastic; however, high bone turnover and consequent excess bone resorption are also characteristic features.

In normal bone, the remodelling process is balanced; however, the presence of tumour cells disrupts the process, causing bone lesions that are detectable by radiographic imaging or radionuclide bone scanning. Lesions result from an imbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation. Osteoclasts adhere to the bone surface and resorb the bone matrix by secreting proteases and acid, and in turn, osteoblasts secrete collagen fibrils that become mineralised, eventually forming bone. However, cross talk between tumour cells and the bone microenvironment causes imbalance in the remodelling process, leading to a pathological “vicious cycle.” Specifically, tumour cells secrete factors that stimulate osteoclast-mediated bone destruction, and factors that were immobilized within the bone matrix are released, stimulating the growth of cancer cells and promoting further bone destruction. Once established in bone, the tumour cells interact with the bone microenvironment in a reciprocal fashion via cytokine mediators to form osteoblastic, osteolytic or mixed lytic/blastic lesions (44-45).

- **Prostate cancer models to target bone metastases**

At present, the molecular mechanism underlying the bone tropism of prostate cancer metastasis is largely unknown. This gap in knowledge is due in part to difficulties in obtaining metastatic tissue from patients, as well as in generating mouse models that display bone metastasis. Experimental models for investigating bone metastases are limited to a small range of xenograft models that typically rely on the intracardiac injection of tumour cells. Prostate cancer cells can also be introduced into the mouse bone marrow through direct inoculation. While these models of human prostate cancer to mouse bone fail to reproduce the early steps of the invasion-metastatic cascade, they often can generate bone metastases at high frequency and have proven to be valuable for elucidating the later steps of this progression. Moreover, these tumours do closely resemble prostate tumours at the histopathologic level that are commonly encountered in the oncology clinic. A list of the models which can be used in preclinical studies targeting the bone is shown in Table II, with the nature of the bone lesions that are generated. In addition, the dynamic monitoring of the metastatic development with multimodality imaging technology such as bioluminescence, SPECT-CT and CT-scan provides a tremendous advantage for studying bone metastases in xenograft models. However, the availability of luciferase-expressing models to realise the follow up of bone metastasis development by BLI is still quite limited. To date there are no genetically modified mouse models that reliably generate bone metastases at an appreciable frequency.

For example, the significant decrease in tumour progression with focal adhesion kinase inhibitor PF562,271 was shown in both the PC3M-luc-C6 subcutaneous local implant and bone metastasis xenograft models, using bioluminescent imaging techniques (59). By using direct intra-tibia injection of PC3 cells, Yang et al (60) showed a reduction of tumour invasion in the bone matrix using the specific Src family kinase inhibitor saracatinib. The PC3-ML cell derivatives were injected directly into the blood system for studying the efficacy of the IMC-3G3 compound targeting PDGFR α on metastatic dissemination (61). With another preclinical model of intracardiac-injected 22Rv1 prostate cancer cells, the efficacy of Atrazentan, an endothelin receptor A antagonist, was evaluated in mixed osteoblastic/osteolytic bone lesions (49).

4. EXPERIMENTAL MODELS TO TARGET RESISTANCE TOWARDS MICROTUBULE-TARGETED CHEMOTHERAPY

- **The use of microtubule-targeted chemotherapy in CRPC and resistance to chemotherapy**

Although only 5% of prostate cancers are diagnosed today with metastases, we know that more than half of high-risk localised prostate cancers will relapse with metastatic lesions. Since 2004, the standard of treatment is chemotherapy with Docetaxel when prostate cancer becomes

resistant to castration. Docetaxel has demonstrated its efficacy with a gain in survival demonstrated in two clinical trials, TAX 327 and SWOG 99-16 (62-63) and is now the treatment of choice in CRPC. Despite the survival benefit afforded by Docetaxel, almost half of patients treated with this agent develop resistance to chemotherapy either immediately or subsequently. Intrinsic and acquired resistances to anticancer agents are the first obstacles to effective cancer therapies. Patients who are refractory to treatment often exhibit multiple resistances to various anticancer agents that may have different structures and different functions. This clinical resistance, comparable to the experimental phenomenon called multidrug resistance (MDR), is probably multifactorial and heterogeneous, involving multiple molecular mechanisms. The mechanisms of this intrinsic or acquired resistance of tumour cells to drugs remain poorly understood and controversial. Efforts should therefore be made to better understand and overcome this serious problem.

- **Cellular mechanism of resistance toward microtubule-targeted drugs**

Microtubules have a diverse set of roles in cellular physiology, including providing structural integrity as part of the cytoskeleton, and a transport system for proteins, vesicles and organelles. They are critically important in the formation of the mitotic spindle, which dictates the proper segregation of chromosomes during mitosis, making them one of the best known targets in cancer therapy. Microtubules are formed by the polymerisation of monomers into linear structures. The combined assembly and disassembly processes yield a state of dynamic instability that allows the growing or shortening of individual microtubules. Vinca-alkaloids bind free β -tubulin monomers, preventing their incorporation into growing microtubules. In contrast, Taxanes and Epothilones bind to β -tubulin already incorporated into microtubules, preventing disassembly, stabilising the mitotic spindle and triggering apoptotic pathways.

Drug resistance in metastatic CRPC is multifactorial and complex. It includes cellular mechanisms of resistance to Taxanes, mechanisms related to the interactions between cancer cells and the surrounding microenvironment and mechanisms related to impaired drug delivery to cancer cells (64). There is also evidence for subpopulations of cancer cells with different proliferative capacity in prostate cancer as cancer stem cells which are considered to be rare multidrug-resistant cells, which repopulate the tumour during and after treatment and may be responsible for treatment failure. At the cellular level, the mechanisms of resistance to Docetaxel and other tubulin-binding chemotherapeutic agents are multifactorial and have been studied in resistant-cell lines and in clinical samples. The mechanisms related to the tubulin and the microtubule systems that mediate resistance to anti-mitotics are complex but they are beginning to be unravelled (65). Overall, Taxanes can be exported outside the cell by the ABC transporters (including ABCB1/MDR1), thus minimising their intracellular concentration and their effect; the expression of tubulin isoforms and microtubule composition may promote the expression of isoforms that are less sensitive to taxanes; mutations of β -tubulin isoforms may be acquired, converting cells into taxane-resistant cells; alterations at mitotic cycle checkpoints may minimise the impact of taxanes on microtubule stabilisation by preventing mitotic catastrophe and apoptotic cell death (65). However, unlike data obtained in breast cancer, the general mechanisms of resistance to Taxanes have not convincingly been demonstrated in prostate cancer. Finally, despite published results on biomarker candidates obtained in small series of tumours, there is still no biomarker predictive of response to Docetaxel for clinical use in prostate cancer. In an effort to identify predictive biomarkers, a standard definition of taxane refractory disease is needed that should enable the possibility of correlating biomarker expression and clinical response to microtubule-targeted chemotherapies.

- **Available models of resistance to microtubule-targeted chemotherapy**

Despite the increasing prevalence of taxane-resistant prostate cancer, there are limited studies in this setting, and very few models of microtubule-targeted chemotherapy resistant prostate cancers are available. Drug-resistant cell lines were generated by exposing the parental

cell line to increasing drug concentrations for a long period. Acquired *in vitro* chemoresistance takes a relatively long time depending of the cell line and must be maintained during successive passages of the drug. Several laboratories have obtained Paclitaxel or Docetaxel-resistant PC3, DU145 and LNCaP derivatives (66-71). In some studies cross-resistance has been assessed (66). The molecular mechanism conferring resistance to docetaxel *in vitro* was shown to be cell-specific (71). Although several drug-resistant cell lines can be generated *in vitro*, the establishment of a drug-resistant model in animals seems very tricky. Docetaxel-resistant PC3 cells were grown in xenografts to study the targeting of clusterin in a docetaxel-refractory prostate cancer preclinical model (72). With another strategy, the efficacy of the combination of estramustine with docetaxel and the appearance of docetaxel resistance was evaluated *in-vivo* in the PAC120 androgen-dependent human xenograft model (73).

5. EXPERIMENTAL MODELS TO TARGET PROSTATE CANCER STEM CELLS

- **The theory of cancer stem cells**

According to the cancer stem cell hypothesis, tumour cells are heterogeneous, and only a subset of cells, known as cancer stem cells (CSCs) are responsible for both self-renewing, giving rise to differentiated cells and producing the remaining cellular heterogeneity represented in the tumour (74). The CSCs exhibit higher resistance to cytotoxic drugs and radiation compared to bulk tumour cells. The survival of few CSCs would result in subsequent tumour regrowth and disease relapse. The concept of cancer as a stem cell disease has the potential to change our view of its treatment. Most therapies are directed at the fast-growing tumour mass but not the slow-dividing cancer stem cells, which may thus explain the frequent ineffectiveness of these therapeutics and suggest that effective therapeutics should target rare CSCs that sustain tumour malignancy. Research is now focusing on understanding the growth of CSCs as well as identifying CSCs antigens in order to discover new targeted approaches. The cancer stem cell hypothesis does not contradict the established clonal evolution view of cancer, but instead suggests that clonal progression of cancer could operate through the stem cell compartment and highlights the importance of an aberrant differentiation programme in tumourigenesis.

- **Application of the CSC hypothesis in prostate cancer**

Evidence for the existence of a stem cell subpopulation in the prostate has been accumulating since the 1980s. There has also been an increasing interest in the role of CSCs in prostate cancer development and progression. However, the cellular origin of CSCs is still controversial. The prostate is comprised of several types of epithelial cells that may serve as targets for cancer initiation, including basal, luminal, and neuroendocrine cells. Normal human prostate stem cells are thought to reside in the basal cell layer of the ducts (75), and several studies have utilised flow cytometry-based approaches to isolate candidate prostate epithelial stem cells directly from primary human tissue and have shown that these stem cells are basal (76). However, a lineage marking methodology has recently been used to identify a rare luminal epithelial population with stem cell properties during prostate regeneration (77).

Prostate cancers have been assumed to originate from luminal cells as prostate cancer is characterised by an expansion of epithelial luminal cells and an absence of epithelial basal cells. The available data on the identity and characterisation of CSCs in localized and advanced prostate cancer were limited. The putative stem cell marker CD133 was shown to isolated prostate cancer cells with stem-cell-like properties *in vitro* (78). Although contested, some studies suggest that normal and prostate cancer stem cells may not express the androgen receptor, implying that prostate cancers may become castration resistant through the survival and expansion of cancer-initiating cells that lack the functional androgen receptor. This theory was confirmed very recently by work performed in a novel human prostate cancer model, showing that only AR-negative basal cells can propagate a tumour which reproduces the histological and molecular features of prostate adenocarcinomas (79).

Future studies are necessary to determine if CSCs are responsible for tumourigenic activity and can be specifically targeted. The goal of existing therapies, such as androgen ablation, has been to eradicate the bulk of cells within a tumour. However by targeting the AR-positive population, resistance occurs in most patients. Mechanisms such as AR amplification do occur, but the resultant tumour may well arise from a more primitive AR-negative clone. We should therefore aim to develop therapeutics that can selectively target CSCs, rather than more differentiated cancer cells. Studying stem cells and their properties will hopefully lead both to a better understanding of cell survival and proliferation in androgen-depleted conditions and to the development of new targets for advanced disease. It is expected that CSCs will be a main target for cancer therapy in the near future.

- **Available PCSC models**

The gold standard assay to demonstrate this concept involves xenotransplantation of flow-sorted populations of primary cancer cells into immunodeficient mice. In this assay, CSCs are defined as a subpopulation of cells within a primary tumour that can initiate tumour formation in mice following transplantation, unlike the remaining tumour cells. Whereas patient samples are the gold standard, they are difficult to obtain regularly. Flow-sorted cells obtained from stable cancer cell lines constitute an alternative and are simple to use but have been selected in a long-term culture. Ectopic xenografts are however limited in analysing CSCs from solid tumours because of the loss of the human stromal microenvironment, which might provide a niche for normal stem cells or for CSCs. Orthotopic models might more faithfully maintain the tumour–host interaction, giving CSCs a greater chance to interact with and obtain stimulation from a mouse environment. The use of more highly immunodeficient mice, orthotopic xenotransplantation and tissue reconstitution with prostate stromal cells may all improve the efficiency and specificity of tumour-initiation assays. The study of CSCs may best be evaluated in xenografts of primary patient isolates that maintain the heterogeneity and architecture of the original patient tumour, but no prostate CSCs have been identified until now using this assay. Avoiding the transplant altogether by generating GEM models of tumours may better maintain the tumour–host and CSC–niche interactions. However, due to the practical difficulty of modelling the multitude of genetic changes in a tumour, mouse prostate cancer models have not been extensively evaluated to date (80).

Cancer stem cell populations are commonly defined by the presence of various combinations of cell-surface proteins, named CSC-markers, and are easily identified and isolated by flow cytometry and/or fluorescence-activated cell sorting (FACS). Isolated CSCs have shown their ability to form spheres in soft agar culture conditions and are more resistant *in vivo* and *in vitro* to common chemotherapeutic drugs due to high expression of drug efflux transporters. Another feature of CSCs is the activation of gene and pathways that are commonly involved in stem cell properties such as the Wnt, Hedgehog and Notch pathways. Thus, prostate cancer cell lines exhibiting these stem-like properties provide cellular models to study the CSC pathways and can be used in xenograft transplantations (Table III). However, it should be kept in mind that, for some of these markers, their specificity for identifying CSC populations has been questioned in some reports, and their biological function is still unknown (80;84).

In this field, PC3 and DU145 xenografts were used to assess the activity of a combination therapy targeting both CSCs and differentiated cell populations in prostate carcinoma (82). It was shown that treatment of prostate tumour xenografts with a combination of the standard chemotherapy (docetaxel) with the NVP-BEZ235 compound targeting CD133+/CD44+ progenitors leads to near complete tumour regression compared to single agent treatments. Thus this study suggests that combination strategies based on bulk tumour reduction and CSC-specific pathway inhibition offer a promising treatment modality. Interestingly, acquired mutations in PTEN appeared selectively in the CD133+/CD44+ cell subset of tumour relapses suggesting that acquired PTEN mutations can serve as a mechanism of drug resistance in CSC (82).

6. EXPERIMENTAL MODELS TO INVESTIGATE THE EFFECT OF RADIOTHERAPY

The aim of curative radiotherapy is to sterilise all clonogenic tumour cells which represent a small subpopulation of all malignant cells in a tumour and may lead to recurrence if they survive following therapy. Whether a tumour can be cured by ionising radiation depends to a large extent on the number and radiation sensitivity of clonogenic tumour cells. The most important treatment parameters that affect local control are the total radiation dose and the toxicity of surrounding normal tissue. However, other factors contribute to the radiotherapy response: cells with the capacity to expand and to form a recurrence, the cancer stem cells, have to be killed. It thus seems important to understand the underlying molecular mechanisms of radiation resistance that give the ability to repopulate to the clonogenic tumour cells, to reoxygenate and to recover from sublethal damage during fractionated irradiation. This should lead to the identification of molecules driving these processes that can constitute new therapeutic targets. It has recently been suggested that CSCs might be more radioresistant than non-tumourigenic cells (93). This issue is of major importance because biomarkers that are under development for predicting the outcome of radiotherapy, including bio-imaging assays, measure the bulk of non-stem cells and not the subpopulation of cancer stem cells.

- **Combining molecular-targeted agents with irradiation in prostate cancer preclinical models**

Combining molecular-targeted agents with irradiation is a promising strategy for patient care. The combinations can be easily studied in preclinical prostate cancer models. In fact, these molecular targets are often differentially expressed in tumours and normal tissue, offering the option of therapeutic gain and they are expressed in only a few tumour stem cells. Endpoints which reflect the killing of CSCs for experiments combining radiotherapy with molecular-targeted drugs using *in vivo* tumour models have been reviewed (94).

The evaluation of irradiation was often done on ectopic xenografts. The combination of radiation with the receptor tyrosine kinase inhibitor AG-013736 was assessed in DU145 prostate cancer cells implanted in the left hind leg of nude mice (95). Multimodality imaging was used in subcutaneous xenografts from luciferase-expressing PC3 cells for the evaluation of ¹⁷⁷Lu-AMBA radionuclide-based therapy (96). As the use of an orthotopic model is essential in prostate cancer because of tumour–stromal interactions, which are particularly important to consider when treatments in combination with androgen deprivation, some studies have evaluated the combination of androgen deprivation and pelvic radiation on intraprostatic xenograft models. The combination of high doses of bicalutamide and radiation was evaluated in orthotopic xenografts from MDA-PCa-2b cells (32), and the effects of antisense-MDM2 on androgen deprivation in combination with radiation was investigated in orthotopic xenografts LNCaP cells (97). In such models tumour response was assessed by magnetic resonance imaging-based tumour volume or human PSA measurements. In order to address the interaction of anti-tumour immune therapy and radiotherapy, the autochthonous murine TRAMP model of prostate cancer has been used to test whether radiotherapy could modify the CD4 T-cell response to a tumour vaccine (98).

7. GENERAL CONCLUSION

The development of better cancer therapeutics continues, and it has become increasingly evident that selection of the appropriate mouse cancer model is essential during the drug discovery process when interrogating novel biological processes in tumourigenesis. Facing continued criticism regarding the predictive value of mouse models, the *in vivo* cancer pharmacologist is challenged to develop models that best enable the study of a specific scientific question. A growing number of research programs are now yielding compounds with demonstrated anti-tumour activity *in vitro* and possible efficacy as *in vivo* agents. The confirmation of therapeutic efficacy therefore requires trials in living, tumour-bearing animals to demonstrate selective anti-tumour activity while remaining safe to non-tumour tissues. Animal models are essential in developing early *in vivo*

biomarkers predictive of therapeutic response of pathway-specific drugs, a field that will see rapid expansion as the costs of undertaking full scale clinical trials escalate. There is a need for improved animal models of human cancer. As our understanding of human disease becomes more comprehensive, the need for more accurate and sophisticated models increases. Experimental therapeutics that target a particular signalling pathway or tumour-specific molecules must be validated in well characterised and suited models that enable the testing of the experimental hypothesis. Sophisticated tumour imaging methodologies are now available to facilitate analysis of anti-tumour efficacy and drug development as bioimaging, use of fluorescent-specific probes, CT- scan as well as technologies such as F-18-labeled compounds followed by PET imaging to selectively label and detect tumour cells and drug biodistribution.

By establishing models that more closely reproduce prostate tumours, and by asking more specific questions with such models, animal models will continue to play an important role in validating cancer targets and evaluating putative cancer therapies. In addition to measuring anti-tumour efficacy, *in vivo* models provide a valuable opportunity to assess pharmacokinetics, pharmacodynamics, drug-drug interactions and preclinical toxicity, as well as initial development of *in vivo* biomarkers as predictors for anti-tumour efficacy. The most important thing is to use the right models to ask the right questions about the right therapies.

CONFLICT OF INTEREST STATEMENT

The new IGR-CaP1 cell line was the subject of an International patent pending entitled "Prostate cancer cell lines and their use in screening method" deposited on the 14th April 2009. Biological material has been deposited at the Pasteur Institut (Paris) (CNCM I-4126). The patented material will be available under a Material Transfer Agreement for research use.

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Name	Origin	Androgen responsiveness	Full-length AR status	AR variants status	In vitro culture	tumourigenicity	Ref.
ARCaP ; ARCaP _M ARCaP _E clones	Ascites fluid of metastatic CRPC	Androgen-repressed	WT LBD	ND	yes	Metastatic dissemination of ARCaP when orthotopically injected; bone and soft tissue metastases	19 ; 20
LNCaP	Lymph node metastase	AS and AI, depending on the passage number or culture conditions	Mutated AR T877A	ND	yes	Weekly tumourigenic, orthotopically injected	21
C4-2	Bone derived subline of LNCaP cells	AI	Mutated AR T877A	ND	yes	S.C. and orthotopic xenografts, metastatic	22
VCaP	Vertebral metastatic lesion of CRPC	AS and AI	WT AR	ARV1, ARV7, ARV8, ARV9, ARV10, ARV11, and other non yet cloned AR variants	yes	S.C. xenograft	23
22RV1	Subline of CWR22 xenograft, originally established from a human prostate primary tumour	AS and AI	Mutated AR: H874Y and in-frame duplication of exon 3	ARV1 to ARV10; AR ^{Ex1/2/2b} and AR ^{Ex1/2/3/2b}	yes	S.C. xenografts	24- 27
LAPC-4	Subline of xenograft derived from advanced adenocarcinoma of the prostate	AS	WT AR	ND	yes	S.C. xenografts	28
MDA-PCa-2a; MDA-PCa-2b	Bone metastases	Low AS	Mutated AR: L701H and T877A	ND	yes	S.C. and orthotopic xenografts; intra-bone injection	29- 32
LuCaP serie (LuCaP 23, LuCaP 35)	LuCaP xenografts were derived from metastases obtained from men with CRPC	AS and AI	AR WT	AR ^{v567es}	no	S.C. xenograft	33- 34
PC346C	Human prostate primary tumour through xenograft	AS	WT AR	ND	yes	S.C. xenografts ; Not tumourigenic when orthotopically implanted	35
KUCaP-1	Human liver metastasis	AS	W741C	ND	no	S.C. xenografts	36- 37
PAC120	Transurethral resection of a locally recurrent prostate cancer	AS	WT AR	ND	no	S.C. xenografts	38

Table I: Commonly used human AR expressing prostate cancer preclinical models

AR: androgen receptor expression; LBD: ligand-binding-domain; AS: androgen sensitive; AI: androgen independent; ND: not determined; S.C.: subcutaneous; WT: Wild-type.

Name	Origin	Androgen responsiveness	In vitro culture	Mode of injection	Type of bone lesions	Ref.
22Rv1	Subline of CWR22 xenograft	Androgen independent	yes	Tail vein injection; intratibia injection ; intracardiac inoculation	Mixed lytic/blastic lesions; spiculated periosteal lesions	46-49
ARCaP; ARCaP _M	Ascites fluid of metastatic CRPC	Androgen-refractory	yes	Orthotopic xenograft; intracardiac inoculation	Osteolytic and osteoblastic lesions	19-20
C4-2B	Subline of LNCaP cells	Androgen-independent	yes	Subcutaneous and orthotopically injected	Osteoblastic lesions	22; 50
IGR-CaP1	Primary prostate cancer	No AR expression	yes	Intratibia injection ; intracardiac inoculation	Mixed lytic/blastic lesions	51; 52
LAPC-9	Xenograft, derived from a metastatic lesion to bone	Androgen-independent	no	Intratibia injection	Bone formation	53-55
MDA-PCa-2b	Bone metastases	Low AS	yes	Intra bone injection	Osteoblastic lesions	29 ; 31 ; 56
PC3	Bone metastase	No AR expression	yes	Intra bone injection ; Intracardiac inoculation	Pure osteolytic lesions	57-61

Table II. Prostate cancer preclinical models to target bone metastases

Name	Origin	Androgen responsiveness	CSC markers expression	Graft sites	Ex vivo expansion	Ref.
PC3	Bone metastase	AR-	PSCA; CD44 ⁺ ; $\alpha 2\beta 1$ -integrin ^{high}	S.C. and prostate, direct intracardiac or intra-tibia-injections	Spheroids; holoclone	81-84
DU145	Brain metastase	AR-	CD44 ⁺ ; CD133 ⁺ CXCR4 ⁺ ; $\alpha 2\beta 1$ integrin ^{high} ; BRCP; CD44 ⁺ CD24 ⁺ $\alpha 2\beta 1$ ⁺	S.C.; bone injection	Holoclone; spheroids	81; 84-88
IGR-CaP1	Primary prostate tumour	AR-	CD44 ⁺ , CD133 ⁺ , CXCR4 ⁺ , $\alpha 2\beta 1$ integrin ^{high}	S.C. and prostate, direct intracardiac or intra-tibia-injections	Spheroids	50
LNCaP	Lymph node metastase	AR+	CD44 ⁺ /CD24 ⁻	S.C. with matrigel	Holoclone	84; 89
22RV-1	Subline of CWR22 xenograft	AR+	CD117 ⁺ /ABCG2 ⁺ ; CD133 ⁺	S.C.; direct intracardiac or intra-tibia-injections	Holoclone	84; 90
LAPC-9	Xenograft	AR+	PSCA; CD44 ⁺ / $\alpha 2\beta 1$ integrin ^{high}	S.C.	Holoclone	91-92

Table III: Models of prostate cancer used to target prostate cancer stem cells.

AR: androgen receptor. Holoclones are round colonies with tightly packed cells that correspond to cells possessing self-renewal stem cell properties (84). Spheroids correspond in 3D structures obtained in anchorage-independent cell culture.