Publisher: Taylor & Francis

Journal: *Expert Opinion on Drug Discovery*

DOI: 10.1080/17460441.2016.1174690

Review:

Challenges with *in vitro* and *in vivo* experimental models of urinary bladder cancer for novel drug discovery

P. A. Oliveira[1,2], R.M. Gil da Costa[5,6], C. Vasconcelos Nóbrega[7,8], R. Arantes-Rodrigues[2,3,4], R. Pinto-Leite[7]

[1] Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[2] Center for Research and Technology of Agro-Environment and Biological Sciences, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5001-801, Vila Real, Portugal

Aveiro, Portugal

[3]QOPNA, Mass Spectrometry Center, Department of Chemistry, University of Aveiro,
[4]Institute for Research and Innovation in Health (I3S), Porto, Portugal

[5]Faculty of Engineering, Laboratory for Process, Environment, Biotechnology and Energy Engineering (LEPABE), University of Porto, Porto, Portugal

[6]Experimental Pathology and Therapeutics Group, Portuguese Institute of Oncology, Rua Dr. António Bernardino de Almeida, Porto, Portugal

[7] Instituto Politécnico de Viseu, Escola Agrária de Viseu, Viseu, Portugal

[8] CECA, Universidade do Porto, Porto, Portugal

[7] Cytogenetic Laboratory, Department of human genetics, Hospital center of Trás-os-Montes and Alto Douro, Vila Real, Portugal

Corresponding Author: PA Oliveira: pamo@utad.pt

Article Highlights Box

- A variety of *in vitro* and *in vivo* models exist for urinary bladder cancer research
- Different models have different uses and limitations: it is therefore very important to choose carefully
- 3D and organotypic cell culture techniques are refining *in vitro* approaches
- A wide-spectrum characterization of available *in vivo* models is needed
- Complementary models provide a comprehensive and realistic view of each problem
- Despite the advances made over the years, there are still many challenges to overcome in in vitro models

Introduction: Urinary bladder cancer (UBC) is the second most frequent malignancy of the urinary system and the ninth most common cancer worldwide, affecting individuals over the age of 65. Several investigations have embarked on advancing knowledge of the mechanisms underlying urothelial carcinogenesis, understanding the mechanisms of antineoplastic drugs resistance and discovering new antineoplastic drugs. *In vitro* and *in vivo* models are crucial for providing additional insights into the mechanisms of urothelial carcinogenesis. With these models, various molecular pathways involved in urothelial carcinogenesis have been discovered, allowing therapeutic manipulation.

Areas covered: This paper provides critical information on existing *in vitro* and *in vivo* models to screen the efficacy and toxicity of innovative UBC therapies and point out the challenges for new and improved models.

Expert opinion: In our opinion, results obtained with *in vitro* and *in vivo* models should be interpreted together, as a set of delicate biological tools that can be used at different stages in the drug discovery process, to address specific questions. With the development of new technologies, new assays and biomarkers are going to play an important role in the study of UBC. The molecular diagnostics and genomic revolution will not only help to develop new drug therapies, but also to achieve tailored therapies.

1 - Introduction

The prognosis and treatment of urinary bladder cancer (UBC) have improved little in the past 20 years. UBC remains a debilitating and often fatal disease, and its treatment is among the most costly [1]. UBC is the second most frequent malignancy of the urinary system and the ninth most common cancer worldwide [2]. Approximately 300.000 individuals are diagnosed each year with UBC worldwide [3]. Involving exogenous and endogenous factors, the aetiology of UBC is multifactorial [4,5], but environmental and occupational exposure to chemical agents, and particularly cigarette smoking, still remain the major risk factors [4].

Since the average life expectancy is increasing, more people are at potential risk of developing UBC. Around eight in ten cases are diagnosed in individuals over the age of 65 and 10% of patients diagnosed with UBC are 85 years old or older [6,7]. The most common cell type of UBC is urothelial cell carcinoma, although adenocarcinomas, squamous cell carcinomas and sarcomas are also identified [8]. The majority of patients with UBC, representing 75-85% of cases, are diagnosed with superficial tumors (low-grade, well-differentiated papillary tumors that do not invade or metastasize), while the remaining 15% to 25% show invasive carcinomas [8,9].

Globally, the incidence rates of UBC vary by geographical regions and ethnicity [10,11]. Racial and ethnic disparities involves Whites, Hispanics, and African Americans. For Hispanics, the incidence of UBC is decreasing [12], and survival rates are similar to Whites [13]. Although incidence rates between African Americans are lower than Whites, African Americans have worse survival, mainly due to their delayed presentation, which manifests as advanced stage and higher grade disease [14-16]. Compared with Whites, African Americans have about a 70% greater risk of cancer- related death [17].

Following transurethral resection of low-grade papillary tumors, adjuvant intravesical instillations of chemotherapy or immunotherapy with Mitomycin C (MMC) and Bacillus Calmette-Guérin (BCG) respectively, are widely applied to decrease the risk of cancer recurrence and progression to a muscle-invasive lesion [18]. The use of BCG not only reduces the recurrence rate, but also reduces the risk of a non-muscle invasive lesion progressing into a muscle-invasive lesion, thus improving the overall chances of survival [19]. Although treatment with BCG provides better results than transurethral resection without immunotherapy, significant side-effects (sepsis, cystitis, dysuria and mild haematuria) may arise from its administration [20]. As experience in using BCG increases, the side-effects now appear to be less prominent [21]. Intravesical chemotherapy with MMC, epirubicin and doxorubicin have all shown comparable beneficial effects [22].

The treatment options currently available for the management of muscle-invasive UBC include radical cystectomy, as well as chemotherapy plus radiation therapy which aim at preserving the urinary bladder. Combined chemotherapy based on methotrexate, vinblastine, adriamycin and cisplatin was initiated in the 1980s, leading to a disease-free survival rate of 3.7% at six years [23]. However, this protocol is highly toxic and has

severe side-effects [24]. Only around 50% of patients with invasive UBC respond to cisplatina based chemotherapy which remains the first-line treatment in different clinical settings: neoadjuvant, adjuvant and metastatic disease [25]. Nevertheless not all patients are eligible for such treatments and long-term survivors are a minority, with 5- year survival rate below 50% for non-metastatic, invasive UBC and 10% for metastatic UBC, with a median survival time of 14 months in this latter group [26]. Thus, there is a clear need for improvement concerning the therapy offered to patients with advanced UBC. UBC is also the malignancy with the highest lifetime treatment cost per patient. These limitations highlight the urgency for the identification and development of new antineoplastic drugs to replace or improve current protocols. In this context, *in vitro* and *in vivo* models are used to better understand UBC behaviour, to assess the effect of chemopreventive agents, to evaluate the mechanism of action of existing drugs, to discover new drug targets and biomarkers and to study the efficacy of new antineoplastic drugs.

Considering the numerous gaps in our knowledge, the aim of this paper is to provide critical information on existing *in vitro* and *in vivo* models to screen the efficacy and toxicity of innovative UBC therapies. In fact, there are many published reports describing the several UBC models available and the drugs already evaluated, however in this review the authors provide now the major *in vitro* and *in vivo* challenges that are necessary overcome in a near future. Overcome these challenges can contribute to a better understanding of tumor behaviour, as well as to develop new and more effective therapies for the treatment of UBC.

2-*In vitro* and *in vivo* experimental models of UBC

2.1 - *In vitro procedures*

In vitro assays using cell lines are used as first-line models in the preclinical development of new drugs to discover, validate, and evaluate the potential of new therapeutic agents [27]. Cancer cell lines have an important role in the study of the biological effects of genetic alterations in different tumor subtypes, and in the identification and characterization of genes involved in cancer initiation and progression [28].

To date, cultured urinary bladder cells correspond to the most commonly used *in vitro* urinary bladder cell model. These models usually consist of isolated UBC cells and have been established as a valid *in vitro* model, to evaluate the efficacy of antineoplastic drugs [29]. In 1970, Rigby and Franks established the first human UBC cell line, designated as RT4 [30]. Since then, several other human UBC cell lines have been established and characterized according to their origin, grade and stage. However, a great part of these cell lines were established from invasive and metastatic tumors, benefiting the investigation of late tumor progression and metastatic lesions. On the other hand, non-muscle-invasive human UBC cell lines available are few, which becomes a disadvantage in when investigating non-muscle-invasive UBC [29]. Available cell lines represent different UBC subtypes and varying degrees of genetic complexity, depending on the sample of origin. However, continuous *in vitro* passages and culture exerts a selective pressure on cells, promoting the acquisition of new mutations and the selection of the fittest cell clones. Several human UBC cell lines have been established and used over the years for numerous purposes, and the genomic and pharmacological profiles of 28 human UBC cell lines are now available in the Cancer Cell Line Encyclopedia (https://www.broadinstitute.org/software/ cprg/?q = node/11) [31].

One of the greatest advantages of *in vitro* models application is the possibility to maintain cells in completely controlled environmental conditions, allowing the study of specific cellular and molecular pathways in shortened experimental timescales. Cell cultures are also less expensive than animal models and less time-consuming, and do not pose identical ethical issues. Conversely, *in vitro* models are severely limited because cells growing *in vitro* are not exact dissociated replicates of their *in vivo* counterparts. The use of monolayer cell cultures is usually restricted to one or two cell types, while tumors are composed not only by neoplastic cells but also by stromal and inflammatory cells, which produce a three-dimensional structure, with critical influence on tumor growth [32]. Also, *in vitro* studies are limited by difficulties in studying tumor immunity, angiogenesis and metastasis, because of the complexity of these processes. *In vitro* studies can provide important information concerning pharmacodynamic parameters, but *in vivo* models are required to study pharmacokinetics, because they currently provide the best approach for analyzing and comparing drug kinetics between different animal species [33]. Likewise, *in vitro* studies do not predict the adverse effects of drugs [34].

In an attempt to circumvent some of these limitations, organotypic and three- dimensional (3D) culture techniques have been developed, which allow the obtention of organoid structures [35]. 3D cell culture systems mimic *in vivo* architecture, cellular heterogeneity, cellular interactions and tumor microenvironmental conditions [36- 38]. These structures may then be assessed histologically and immunohistochemicaly for the expression differentiation-related and other molecules, providing a model for studying urothelial development, neoplasia and reconstruction.

Normal urothelial cells may be readily cultivated *in vitro* using monolayer cultures for use as controls, and culture techniques have been long established [39]. The seeding of

normal urothelial cells over stromal matrices yields an organoid structure, which recapitulates the histological and molecular features of normal urothelium [40]. When the stromal matrix is seeded with urothelial cancer cell lines, these display an invasive behaviour, which again may be appreciated histologically and immunohistochemically [41]. This approach allowed the elucidation of important aspects of molecular cross-talk in normal and neoplastic urothelium [42]. Different stroma matrices may be used, including engineered materials [43], and used to assess epithelial-stroma interactions [44, 45].

The organ-on-a-chip approach adds further complexity to these model systems, allowing for the production of over 1.0 cm-thick organoids with customized vasculature and stroma [46]. This approach allows researchers to accurately model tissues on a microscale, including the modelling of complex organs like the urinary bladder [47], holding great promise for cancer research.

In the last thirty years, numerous *in vitro* studies were conducted to evaluate the activity of antineoplastic drugs, making use of several human UBC cell lines and a wide range of methodologies. As an example, the cell lines 5637, T24 and HT1376 were used to evaluate the effect of everolimus and temsirolimus [sirolimus analogs and mammalian target of rapamycin (mTOR) inhibitors] in UBC cells. mTOR signaling was found to play an important role in cell growth, survival, proliferation, and angiogenesis in many solid tumors including UBC, where it is believed to have potential for prognostic information and targeted therapy [28]. According to these studies, sirolimus analogs exert a slight interference on proliferation, apoptosis, and autophagy in these cancer cell lines. The non-muscle invasive UBC cell line 5637 was the most sensitive to mTOR inhibitor treatment alone [48,49]. These researchers further evaluated the combined effect of mTOR inhibitors with gemcitabine and cisplatin, which resulted in enhanced

inhibition of cell proliferation and increased apoptosis and autophagy, especially in 5637 and HT1376 cells. In contrast, in the T24 cell line, everolimus or temsirolimus enhanced gemcitabine but not cisplatin efficacy [50-53]. Additional in-depth mechanistic studies to elucidate the interactions between antineoplastic drugs and their targets are feasible *in vitro*, but *in vivo* models are needed to advance the following stages of pre-clinical drug development.

2.2- *In vivo procedures*

In vivo models of UBC are described as valid if they resemble the human condition in aetiology, pathophysiology, symptomatology, target identification, and response to therapeutic interventions [54].

Most of the time, researchers focus on experimental *in vivo* models established in laboratory rodents, forgetting the spontaneous models of disease. However, spontaneous animal models of UBC are available and present important opportunities for research.

2.2.1- Spontaneous animal models of UBC

Comparative oncology's contributions to the field of cancer biology and anticancer drug development are now fully recognized [55]. The spontaneous UBC model that recapitulates the human disease more closely occurs in the dog. Although the true incidence of canine TCC is not known, TCC is the most common form of urinary tract cancer in the dog and comprises 1.5 to 2% of all canine cancers [56,57]. The advantages of this model include: (1) similarities between canine and human bladder cancer in histopathologic appearance, biological behaviour, and response to therapy and prognosis, (2) similar drug metabolism between dogs and humans, (3) less constraints in testing new therapies, since it does not exists a standard therapy for many canine

cancers, (4) the compressed lifespan of the dog, which makes completion of clinical studies possible in a timely manner, (5) the fact that dogs share the environment with their owners and thus have similar exposures to water, passive cigarette smoke, and insecticides, (6) the larger size of the dog compared with other animal models like rodents, which makes many medical procedures technically feasible, and (7) the concept that novel interventional strategies developed *in vitro* or in laboratory animal studies can be tested *in vivo* in spontaneous tumor-bearing dogs, before being applied to humans [58-63]. UBC studies in dogs also have the potential to define heritable and environmental risks, methods to detect UBC earlier, and methods to more effectively treat UBC, important information to better understand, prevent and treat the disease in their human's counterparts. Compared with humans, TCC in dogs can be low-grade with a superficial papillary appearance or be high-grade invasive tumors that spread through the urinary bladder wall to lymph nodes and to other organs, predominantly the liver and lung [56,62]. Although low-grade, superficial TCC comprises the majority of TCC in humans, it is very uncommon in dogs [57]. Other of the few differences between canine and human TCC is in gender predilection, with men being at greater risk that women, and female dogs being at greater risk than male dogs [57,61]. Another spontaneous UBC model is found in cattle exposed to the poisonous fern *Pteridium* spp. and its toxin, ptaquiloside [reviewed by 64]. However, the urinary bladder lesions observed in this model, and in experimental models established using the same toxins, are histologically heterogeneous and differ too much from those found in human patients [64-66] to allow the use of this model for drug development.

The use of spontaneous animal models of disease circumvents the ethic concerns related to the use of laboratory animals: it is more acceptable to a society concerned with animal welfare to treat sick animals and use the knowledge obtained in favor of

scientific development, than inducing disease in animals for research. Moreover, any comparative studies performed in pet animals clearly benefit both animals and humans [56, 63].

2.2.2- Experimental models of UBC

The first experimental animal model used to study UBC was reported by Hueper, in 1938. This experiment used 2-naphthylamine to induce UBC in dogs [67]. Since then, various attempts to induce tumors in rodent urinary bladders by chemicals were unsuccessful until 1944, when Armstrong and Bronser induced papillomas and carcinomas through oral administration of 2-acetylaminoflourene (AAF) in CBA strain mice [68]. This team induced urinary bladder papillomas and carcinomas in CBA mice with the chemical carcinogen 2-acetylaminofluorene (AAF). Although of considerable usefulness in experimental urinary bladder carcinogenesis, AAF is a pluripotent carcinogen, which means this compound also induces tumors in other organs of laboratory rodents (liver, pancreas, breast, skin, forestomach and ear duct). In the following decades, several researchers focused their search for urinary bladder-specific carcinogens. During the 1960s, three compounds in particular were found to have some specificity for the urothelium: *N*-[4-(5-nitro-2-furyl)-2 thiazolyl]formamide (FANFT), *N*-methyl-*N*-nitrosurea (MNU) and *N*-butyl-*N*-(4-hydroxybutil) nitrosamine (BBN).

FANFT and MNU were long used to induce UBC in laboratory animals, but they are seldom used presently because both have very particular handling requirements. FANFT is administered in the animals' diet and is a dangerous compound that must be manipulated with care due to its carcinogenic and teratogenic risks for reseachers [69]. MNU is a direct- and complete-acting carcinogen causing tumor initiation and/or promotion not only in the urinary bladder but also in mammary gland, lungs, liver,

thyroid, pancreas, prostate, intestine, forestomach, glandular stomach, alimentary tract, kidney, nervous system and hematopoietic system [70]. Tumor location depends on the route of MNU administration, as well as on the species, age and sex of the animals. In order to induce UBC, MNU must be instilled intravesically, a technical requirement which isn't easily met by most research teams [71]. Because MNU is intrinsically unstable, variations in carcinogenic potency can arise, leading to inconsistent data, unless care is taken during its storage, preparation and use [72]. Currently, BBN is the most used urinary bladder carcinogen *in vivo*, since its carcinogenic potential is essentially limited to this organ [73]. BBN may be easily administered in drinking water at a dose ranges from 0.01 to 0.05%, however the use of opaque bottles is necessary, since this is a photosensitive compound [74]. This compound may also be administered by other routes: oral gavage, subcutaneous injections and intravesical instillation [75]. Each of these routes of administration has advantages and disadvantages that should be considered, but frequently, the team's prior experience determines the route of choice. Oral gavage requires specific training of the staff involved while intravesical instillations require the use of female animals following animal sedation [76,77]. As with MNU, the incidence of urothelial lesions induced by BBN depends on the route of administration and the incubation period following carcinogenic exposition [78,79].

In early works of urinary bladder carcinogenesis, researchers were concerned to classify histopathologically all lesions induced, and to compare them with those described in human patients, which was critical to validate their animal models. These studies soon showed that BBN induced papillary UBC in rats and invasive and papillary UBC in mice, morphologically similar to those observed in human patients [80,81]. Accordingly, such models were used for preclinical drug development early on, and were later subjected to immunohistochemical evaluation, revealing further similarities

with their human counterparts [82]. In the present, the use of chemically-induced models are limited by the costs involved, the long experimental protocols, difficulties in monitoring UBC development during the experimental protocol, and the fact that their molecular characteristics remain only partially understood [28,69]. Application of genomic, transcriptomic, proteomic and other wide-spectrum techniques to these models will help refining our understanding of their underlying molecular changes and re-assess their usefulness for translational research.

Transplantation models offer another strategy for *in vivo* cancer research. Transplantation methods comprise various systems and techniques to propagate tumor cells in different hosts for controlled studies *in vivo*. Some of these methods have been used for decades and are well-established, while others are more recent and continue to develop. These models may be classified as allografts, also known as syngeneic, or as xenografts. A syngeneic model consists of rodent UBC cells or tumor fragments transplanted into an immunocompetent host of the same species and strain, allowing researchers to monitor tumor growth and other parameters. Xenograft models consist of human cancer cells or tumor fragments transplanted into immunodeficient hosts, most usually mice [83]. Both syngeneic and xenograft models have advantages and drawbacks to be considered. Syngeneic models take advantage of a fully-functional host immune system (as opposed to the immunodeficient xenografted animals), while xenografts make use of human rather than murine cells.

Tumor transplants may be orthotopic, meaning that the tumor is placed in the site it would be expected to arise naturally in the host; or they may be heterotopic, which means that the transplants are placed in other locations, most often subcutaneously. Orthotopic tumor models have the great advantage of simulating the local cancer environment and recapitulating the natural history of the disease [83-85]. In general, an

orthotopic, immunocompetent model has advantages over models that use immunodeficient rodents and/or heterotopic locations [86], although it's more difficult to perform. To implement orthotopic models additional methodologies are previously required. A chemical urothelial denudation (leading to multifocal lesions) or a mechanical urothelial lesion (requiring cystotomy), is necessary before cells can be introduced into the urinary bladder cavity [87,88]. Also a number of factors can affect the tumor take rate, such as the amount of UBC cells instilled, the UBC cell concentration, the instillation volume and the tumor cell dwell time in urinary bladder, reasons for why results obtained not always are reproducible [89]. Furthermore, it is also important to point out that intravesical drug instillation in laboratory rodents is only possible in females; anatomical differences render this technique impossible in males [77].

Heterotopic tumor models have been widely used, because subcutaneous implantation is commodious and facilitates tumor follow-up [83-85], but their different microenvironmental conditions may limit correlations with natural disease. Particularly in the case of urinary bladder, the heterotopic model presents another disadvantage since the implanted tumor cells are not exposed to the urinary bladder, which could contribute to the kinetics of the drugs under evaluation, as well as to the tumor behaviour. Besides, with both heterotopic and orthotopic models, there may be a long latency period before tumours becomes noticeable and the take rate is generally low when passaging tumor samples for the first time.

Current transgenic UBC models aim to circumvent some of these limitations and several genetically modified animal strains are available, to be selected for the purposes of each specific study [90-93], as reviewed by Oliveira *et al*., (2006) [69]. For instance, expression of the simian virus 40 large T antigen has been targeted to the urothelium

using the uroplakin II gene promoter in a tumor-permissive FVB/n background [91]. These UPII-SV40 mice develop invasive UBC and became useful and popular immunocompetent models for studying the pathogenesis of these lesions and testing innovative therapies [94].

The use of animal models enabled the identification of critical signalling pathways involved in urinary bladder tumorigenesis and of valid therapeutic targets, such as the PI3K/AKT/mTOR and the RAS-MAPK pathways [95-97]. Endothelial growth factors which promote angiogenesis are also possible targets [98,99]. Animal models are critical for modern lineage-tracing studies which identify specific urothelial cell populations as the origin of certain kinds of UBC [100,101].

Among therapeutic agents targeting the PI3K/AKT/mTOR pathway, mTOR inhibitors are the most developed ones. Rapamycin, also known as sirolimus, and its derivatives such as everolimus, tensirolimus and deforolimus, have antineoplastic activity when it comes to a variety of solid tumors - including UBC - and are being tested in clinical trials. Some of these drugs were initially tested pre-clinically *in vivo* [102-104]. Other agents like endostatin, bevacizumab and cetuximab that can target vascular endothelial growth factor and, therefore, tumor angiogenesis are also being subjected to clinical trials [105,106]. Cetuximab and panitumumab also target EGFR and HER2/*neu,* two generally upregulated genes [105,109]. Tipifarnib, a farnesyltransferase inhibitor, is able to inhibit farnesylation, an important step connected with *Ras* activation. Sorafenib (BAY43-9006) is an oral, dual inhibitor of *Raf* and vascular endothelial growth factor receptors (VEGFR). Other agents that have shown promising results regarding UBC (vinflunine, celecoxib, TKI-258 and AEZS-108), are currently under clinical trials [106,108,109]. This entire therapeutic advance would not be possible without *in vivo* models, but it must be remembered that results obtained in experimental animal models

cannot be directly translated into clinical practice, although they provide important information regarding drug delivery, pharmacokinetics and potential toxicity.

3- CONCLUSIONS

The development of realistic *in vitro* and *in vivo* models has been a major concern of the scientific community, in order to build a strong and reliable body of evidence for use in the field of oncology. The time and money invested through the years produced better models and it is now easier to conduct more sophisticated research. However, there is no perfect model. If on the one hand, *in vitro* and *in vivo* models are specific, very well characterized, and have considerably advanced our understanding on UBC, on the other hand they show constraints which must be considered for their successful use in drug discovery, in the clarification of the oncogenic process and in the reaction to specific therapeutic agents. For that reason, all the information obtained with different models should be analysed as a whole and with caution. It is not wise nor expected to extrapolate experimental preclinical data as a complete predictor of the human reaction to a novel therapeutic agent. Results obtained with *in vitro* and *in vivo* models should be interpreted together, as a set of delicate biological tools that can be used at different stages in the drug discovery process, to address specific questions. With the development of new technologies and innovations, new assays and biomarkers are going to play an important role in the study of UBC. This development is being driven by molecular diagnostics and the genomic revolution that will not only help to promote the development of new drug therapies, but also to achieve a tailored therapy.

4- EXPERT OPINION

Studies conducted in the past decades on experimental models (*in vitro* and *in vivo*) have provided the basis for most of our current knowledge on the pathophysiological mechanisms of UBC. Nevertheless, a considerable hiatus remains between the advances made in understanding pathophysiological mechanisms and the identification of new and effective drugs to treat UBC. Although many *in vitro* and *in vivo* studies have been carried out in the last four decades and promising targets have been identified, only a few (sirolimus, gemcitabine, cysplatin, atezolizumab; ramucirumab, pazopanib, nivolumab, among others) compounds have reached clinical trials. This problem may be due, at least in part, to the lack of well-established procedures and screening protocols to evaluate anticancer activity of a given compound and to the lack of cancer markers for preclinical assays. And, for that reason, the UBC protocol treatments remain the same since the 1980s. Therefore, the development and screening of antineoplastic drugs will require the standardization of assays to a general format that will be used to evaluate the efficacy of novel compounds in an optimized, safe and reproducible manner. Relevant aspects of UBC biopathology shall be considered, in order to establish a minimum set of criteria and decision gates.

Cancer cell lines play a major role in research, especially through gene expression manipulation to introduce gain- or loss-of-function alterations [28]. The development of 3D and organotypic tissue culture techniques already allows researchers to explore microenvironmental interactions *in vitro* which affect angiogenesis and tumor invasion [35,110]. Currently, there are approximately 40 urinary bladder cell lines and commercial companies dispose them with severe controls of integrity. However, after several culture passages, a different genetic profile may develop and cross-contaminated or misidentified cell lines may arise, making cell line authentication mandatory for such

studies. At the moment, it is considered safe to use a cell line during five years or thirty passages. As a rule, cell lines without an authentication certificate should not be used.

Standard cell culture studies are widely used to delineate the biological, chemical and molecular cues of living cells. UBC cell lines established from human tumors of different stages and grades, allow us to understand the heterogeneous response observed by the same drug between patients. However, three-dimensional models that recapitulate the tumor microenvironment remain essential. While 3D culture systems and organotypic cell cultures develop, researchers necessarily turn to i*n vivo* models.

In developed countries, the etiological factors of UBC are genetic predisposition and chemical exposure. Most of the current models only take into account one of these factors and therefore do not reproduce the complexity of UBC. Animal models are most suited for the combined analysis of these factors and the evaluation of their exact contribution to the development of the pathology. Other etiologic agents, namely *Schistosoma haematobium*, were also implicated in the development of UBC, particularly in the Nile River Valley in Africa. Despite the numerous existing UBC models, the pathophysiology of UBC associated with this parasite remains unknown. This is mainly due to the lack of a tractable animal model. Hsieh *et al*., have developed a mouse model of *S. haematobium* urinary tract infection after microinjection of purified *S. haematobium* eggs into the urothelial bladder wall [111]. This model recapitulates several aspects of human urothelial schistosomiasis but the development of infection-associated UBC was not reported and remains to be explored [28]. Given the suitability of the canine model for UBC research, it seems natural to take advantage of its wide occurrence in order to advance translational research. A cross-species comparative study of available models is now in order, so as to identify those animal models most suited to each particular purpose.

Nowadays, genetic engineered animals are among the most popular animal models for the study of UBC. However, such models do not necessarily reproduce the heterogeneity of human UBC. Thus, research should also be carried out in alternative models, probing each problem from different angles. In fact, reasons for the unsuccessful translation of preclinical research into clinical application may include limited knowledge of the specific characteristics of the animal models available. The development of novel *in vitro* and *in vivo* technologies progressively permits the execution of more refined and complex experiments, while facilitating result interpretation. Conditional transgenic mouse models allow researchers to control the activation of multiple oncogenes, pointing the way forward and providing critical insights into questions that were, until recently, very difficult to address.

The development of immunological checkpoint inhibitors for treating UBC also requires a careful choice of adequate models. Using monoclonal antibodies to block immunological checkpoint molecules and boost anti-tumor immune responses is a promising new strategy, first developed against melanoma [112]. Two checkpoint inhibitors are currently in clinical trials against urothelial carcinoma (atezolimumab, which inhibits programmed death ligand 1 - PD-L1 - and ipilimumab, a cytotoxic T lymphocyte antigen 4 - CTLA-4 - inhibitor) [113]. Most immunocompetent mouse and rat strains should be suitable for developing new immune checkpoint therapies, and anti-CTLA-4 or anti-PD-L1 therapies were already studied using a syngeneic mouse C57Bl/6 model based on MB49 urothelial carcinoma cells [114].

Despite the advances made over the years, there are still many challenges to overcome in *in vitro* models, namely: the cytogenomic, genomic, epigenomic, transcriptomic and proteomic characterization of UBC cell lines; improve 3D and organotypic tissue culture techniques to enhance the study of UBC angiogenesis and the role of

microenvironmental immunity; and identify culture conditions that maintain the morphology, genomic profile, gene expression and signaling pathways of cultivated UBC cells for extended periods. Also the *in vivo* models has several challenges that are extremely important to overcome, such as the identification of reliable early diagnostic and progression markers of UBC in animal models, preferably coupled with non- invasive methodologies, a comprehensive cytogenomic, genomic, epigenomic, transcriptomic and proteomic characterization of UBC animal models, cross-species study of the morphological and molecular features of UBC in available animal models and human patients and further identification of drug targets and effective therapies.

Declaration of Interest

This work is supported by national funds by FCT - Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013 and POCI/01/0145/FEDER/006958. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Kobayashi T, Owczarek TB, McKiernan JM, Abate-Shen C. Modelling bladder cancer in mice: opportunities and challenges. Nature Reviews Cancer 2015;15:42-54

• Review on the currently mice models of urinary bladder cancer.

2. Babjuk M, Burger M, Zigeuner R, Shariat SF, et al. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. Eur Urol 2013;64:639-53

3. Jemal A, Siegel R, Ward E, Hao Y, et al. Cancer statistics, 2009. CA Cancer J Clin 2009;59:225-49

4. Tanaka MF, Sonpavde G. Diagnosis and management of urothelial carcinoma of the bladder. Postgrad Med 2011;123(3):43-55

5. Botelho MC, Machado JC, Brindley PJ, Correia da Costa JM. Targeting molecular signaling pathways of Schistosoma haemotobium infection in bladder cancer. Virulence 2011;2(4):267-79

6. Racioppi M, Palermo G, D'Addessi A, Pinto F, et al. Hot topics in urological health economics. A mini review. Arch Ital Urol Androl 2012; 84:47-52

7. Lopez-Beltran A, Sauter G, Gasser T, et al. Infiltrating urothelial carcinoma World Health Organization Classification of Tumours Pathology and Genetics of Tumours of the Urinary System and Male Genital System. Lyon: France, 2004. p. 93-109

8. Kompier LC, Lurkin I, van der Aa MNM, van Rhijn BW, et al. FGFR3, HRAS, KRAS, NRAS and PIK3CA mutations in bladder cancer and their potential as biomarkers for surveillance and therapy. Plos One 2010;5(11): e13821

9. Cohen SM. Comparative pathology of proliferative lesions of the urinary bladder. Toxicol Pathol 2002;30(6):663-71

• This paper compares urothelial lesions identified in rodents after chemical carcinogens exposition.

10. van Rhijn BW, Burger M, Lotan Y, et al. Recurrence and progression of disease in non-muscle-invasive bladder cancer: from epidemiology to treatment strategy. Eur Urol 2009;56(3):430-42

11. Abdulamir AS, Hafidh RR, Kadhim HS, Abubakar F. Tumor markers of bladder cancer: the schistosomal bladder tumors versus non-schistosomal bladder tumors. J Exp Clin Cancer Res 2009;28:27

12. Canto MT, Chu KC. Annual cancer incidence rates for Hispanics in the United States: Surveillance, epidemiology, and end results, 1992–1996. Cancer 2000;88:2642- 52

13. Fleshner NE, Herr HW, Stewart AK, et al. The National Cancer Data Base report on bladder carcinoma. The American College of Surgeons Commission on Cancer and the American Cancer Society. Cancer 1996;8:1505-13

14. Underwood W III, Dunn RL, Williams C, et al. Gender and geographic influence on the racial disparity in bladder cancer mortality in the US. J Am Coll Surg 2006;202:284-90

15. Prout GR Jr, Wesley MN, McCarron PG, et al. Survival experienceof black patients and white patients with bladder carcinoma. Cancer 2004;100:621-30

16. Scosyrev E, Noyes K, Feng C, et al. Sex and racial differences in bladder cancer presentation and mortality in the US. Cancer 2009;115:68-74

17. Bach PB, Schrag D, Brawley OW, et al. Survival of Blacks and Whites after a cancer diagnosis. JAMA 2002;287:2106-13

18. Wirth M, Plattner VE, Gabor F. Strategies to improve drug delivery in bladder cancer therapy. Expert Opin Drug Deliv 2009;6:727-44

19. Tanaka MF, Sonpavde G. Diagnosis and management of urothelial carcinoma of the bladder. Postgrad Med 2011;123:43-55

20. Lamm DL. Complications of bacillus Calmette-Guerin immunotherapy. Urol Clin North Am 1992;19:565-72

• Paper that describe the secondary effects of Bacillus Calmette-Guérin intravesical instillation in urinary bladder cancer treatment.

21. van der Meijden AP, Sylvester RJ, Oosterlinck W, et al. Maintenance Bacillus Calmette-Guerin for Ta T1 bladder tumors is not associated with increased toxicity: Results from a European Organisation for Research and Treatment of Cancer Genito-Urinary Group Phase III Trial. Eur Urol 2003;44:429-34

22. Sylvester RJ, Oosterlinck W, van der Meijden AP. A single immediate postoperative instillation of chemotherapy decreases the risk of recurrence in patients with stage Ta T1 bladder cancer: A meta-analysis of published results of randomized clinical trials. J Urol 2004;171:2186-90

23. Ismaili N, Amzerin M, Flechon A. Chemotherapy in advanced bladder cancer: Current status and future. J Hematol Oncol 2011;4:35-45

• Review on the currently treatments of urinary bladder cancer.

24. Loehrer PJS, Einhorn LH, Elson PJ, et al. A randomized comparison of cisplatin alone or in combination with methotrexate, vinblastine, and doxorubicin in patients with metastatic urothelial carcinoma: A cooperative group study. J Clin Oncol 1992;10:1066-73

25. Hafeez S, Horwich A, Omar O, et al. Selective organ preservation with neo-adjuvant chemotherapy for the treatment of muscle invasive transitional cell carcinoma of the bladder. Br J Cancer. 2015;112:1626-1635

26. Stenzl A, Cowan NC, De Santis M, et al. Treatment of Muscle-invasive and Metastatic Bladder Cancer: Update of the EAU Guidelines. Eur Urol. 2011;59:1009-1018

•• This paper reports the EAU guidelines to treat urinary bladder cancer.

27. Monks A, Scudiero D, Skehan P, et al. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J Natl Cancer Inst 1991;83:757-66

28. Bernardo C, Costa C, Palmeira C, et al. What we have learned from urinary bladder cancer models. J Cancer Metastasis Treat 2016; [Epub ahead of print]

29. Gabriel U, Bolenz C, Michel MS. Experimental models for therapeutic studies of transitional cell carcinoma. Anticancer Res 2007;27:3163-71

30. Rigby CC, Franks LM. A human tissue culture cell line from a transitional cell tumour of the urinary bladder: growth, chromosone pattern and ultrastructure. Br J Cancer 1970;24(4):746-54

31. Barretina J, Caponigro G, Stransky N, et al. The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature 2012;483:603-7

32. Hadaschik BA, Black PC, Sea JC, et al. A validated mouse model for orthotopic bladder cancer using transurethral tumour inoculation and bioluminescence imaging. BJU Int 2007;100:1337-48

33. Jones RF, Debiec-Rychter M, Wang CY: Chemical carcinogenesis of the urinary bladder - a status report. J Cancer Res Clin Oncol 1992;118:411-19

34. Cohen SM. Role of cell proliferation in regenerative and neoplastic disease. Toxicol Lett 1995;82-83:15-2

35. Varley CL, Southgate J. Organotypic and 3D reconstructed cultures of the human bladder and urinary tract. Methods Mol Biol 2011;695:197-211

36. Thoma CR, Zimmermann M, Agarkova I, et al. 3D cell culture systems modeling tumor growth determinants in cancer target discovery. Adv Drug Deliv Rev 2014;69-70:29-41

37. Kimlin L, Kassis J, Virador V. 3D in vitro tissue models and their potential for drug screening. Expert Opin Drug Discov 2013;8(12):1455-66

38. Weiswald LB, Bellet D, Dangle-Marie V. Spherical cancer models in tumor biology. Neoplasia 2015;17(1):1-15

39. Southgate J, Hutton KA, Thomas DF, et al. Normal urothelial cells in vitro: proliferation and induction of stratification. Lab Invest 1994;71:583-94

40. Scriven SD, Booth C, Thomas DF, et al. Reconstitution of human urothelium from monolayer cultures. J Urol 1997;158:147-1152

41. BoothC, Harnden P, Trejdosiewicz LK, et al. Stromal and vascular invasion in an human in vitro bladder cancer model. Lab Invest 1997;76:843-57

42. Booth C, Hsrnden P, Selby PJ, et al. Towards defining roles and relationships for tenascin-C and TGF beta-1 in the normal and neoplastic urinary bladder. J Pathol 2002;198:359-68

43. Scriven SD, Trejdosiewicz LK, Thomas DF, et al. Urothelial cell transplantation using biodegradable synthetic scaffolds. J Mater Sci Mater Med 2001;12:991-6

44. Dabelsteen S, Hercule P, Barron P, et al. Epithelial cells derived from human embryonic stem cells display p16INK4A senescence, hypermotility and diferentiation properties shared by many P63+ somatic cell types. Stem Cells 2009;27:1388-99

45. Mudge CS, Klumpp DJ. Induction of the urothelial differentiation program in the absence of stromal cues. J Urol 2005;174:380-5

46. Kolesky DB, Homan KA, Skylar-Scott MA, et al. Three-dimensional bioprinting of thick vascularized tissues. Proc Natl Acad Sci USA 2016; pii:01521342

47. Vrana NE, Lavalle P, Dokmeci MR, et al. Engineering functional epithelium for regenerative medicine and in vitro organ models: a review. Tissue Eng Pat B Rev 2013;19:529-43

48. Vasconcelos-Nóbrega C, Pinto-Leite R, Arantes-Rodrigues R, et al. In vivo and in vitro effects of RAD001on bladder cancer. Urol Oncol 2013;31:1212-21

49. Pinto-Leite R, Botelho P, Ribeiro E, et al. Effect of sirolimus on urinary bladder cancer T24 cell line. J Exp Clin Cancer Res 2009;28:3

50. Pinto-Leite R, Arantes-Rodrigues R, Palmeira C, et al. Everolimus combined with cisplatin has a potential role in treatment of urothelial bladder cancer. Biomed Pharmacother 2013;67:116-21

51. Pinto-Leite R, Arantes-Rodrigues R, Palmeira C, et al. Everolimus enhances gemcitabine-induced cytotoxicity in bladder-cancer cell lines. J Toxicol Environ Heal Part A 2012;75:788-99

52. Pinto-Leite R, Arantes-Rodrigues R, Ferreira R, et al. Temsirolimus improves cytotoxic efficacy of cisplatin and gemcitabine against urinary bladder cancer cell lines. Urol Oncol Semin Orig Investig 2014;32:41.e11-22

53. Pinto-Leite R, Arantes-Rodrigues R, Ferreira R, et al. Treatment of muscle invasive urinary bladders tumors: a potential role of the mTOR inhibitors. Biomed Aging Pathol 2014;4:169-78

54. Van Dam D, De Deyn PP. Drug discovery in dementia: the role of rodent models. Nature Reviews Drug Discovery 2006;5,956-70

55. LeBlanc AK. Cancer and comparative imaging. ILAR J 2014;55(1):164-8

56. Knapp DW, Glickman NW, De Nicola DB, et al. Naturally-occurring canine transitional cell carcinoma of the urinary bladder - A relevant model of human invasive bladder cancer. Urologic Oncology 2000;5:47-59

57. Knapp DW, Ramos-Vara JA, Moore GE, et al. Urinary bladder cancer in dogs, a naturally occurring model for cancer biology and drug development. ILAR J 2014;55(1):100-18

58. Henry CJ, McCaw DL, Turnquist SE, et al. Clinical evaluation of mitoxantrone and piroxicam in a canine model of human invasive urinary bladder carcinoma. Clin Cancer Res 2003;9(2):906-11

59. Glickman LT, Raghavan M, Knapp DW, et al. Herbicide exposure and the risk of transitional cell carcinoma of the urinary bladder in Scottish Terriers. J Am Vet Med Assoc 2004;224(8):1290-97

60. Shearin AL, Ostrander EA. Leading the way: canine models of genomics and disease. Disease Models & Mechanisms 2010;3:27-34

61. Knapp DW, Waters DJ. Naturally occurring cancer in pet dogs: important models for developing improved cancer therapy for humans. Mol Med Today 1997;3(1):8-11

62. Cekanova M, Rathore K. Animal models and therapeutic molecular targets of cancer: utility and limitations. Drug Des Devel Ther 2014;14(8):1911-21

63. Lairmore MD, Khanna C. Naturally occurring diseases in animals: contributions to translational medicine. ILAR J. 2014;55(1):1-3

64. Gil da Costa RM, Bastos MMSM, Oliveira PA, Lopes C. Bracken-associated human and animal health hazards: chemical, biological and pathological evidence. Journal of Hazardous Materials 2012;203-204:1-12

65. Gil da Costa RM, Oliveira PA, Vilanova M, et al. Ptaquiloside-induced B-cell lymphoproliferative and early-stage urothelial lesions in mice. Toxicon 2011;58:543- 549

66. Gil da Costa RM, Oliveira PA, Bastos MMSM, et al. Ptaquiloside-induced early-stage urothelial lesions: increased cell proliferation and intact ² -catenin and E-cadherin expression. Environmental Toxicology 2014;29:763-69

67. Hueper WC, Wiley FH, Wolfe HD, et al. Experimental production of bladder tumors in dogs by administration of beta-naphthylamine. J Ind Hyg Toxicol 1938;20:46-84

68. Armstrong EC, Bonser GM. Epithelial tumours of the urinary bladder in mice induced by 2-acetylamino-fluorine. J Pathol 1944;6:506-12

69. Oliveira PA, Colaco A, De la Cruz PLF, et al. Experimental bladder carcinogenesis-rodent models. Exp Oncol 2006;28:2-11

70. Lijinsky W. Species differences in nitrosamine carcinogenesis. J Cancer Res Clin Oncol 1984;108:46-55

71. Steinberg GD, Brendler CB, Ichikawa T, et al. Characterization of an N-methyl-N-nitrosourea-induced autochthonous rat bladder cancer model. Cancer Res 1990;50:6668-74

72. Kunze E, Chowaniec J. Pathology of tumours in laboratory animals. Tumours of the rat. Tumours of the urinary bladder. IARC Sci Publ 1990;99:345-97

73. Oliveira PA, Palmeira C, Lourenço L, et al. Evaluation of DNA content in preneoplastic changes of mouse urinary bladder induced by N-butyl-N-(4- hydroxybutyl) nitrosamine. J Exp Clin Cancer Res 2005;24:207-14

74. Ito N, Shirai T, Fukushima S, et al. Dose-response study of urinary bladder carcinogenesis in rats by N-butyl-N-(4-hydroxybutyl)nitrosamine. J Cancer Res Clin Oncol 1984;108:169-73

75. Cohen SM, Friedell GH. In: The mouse in biomedical research. Neoplasms of the urinary system. New York: Academic Press, 439–63, 1982

76. Arantes-Rodrigues R, Henriques A, Pinto-Leite R, et al. The effects of repeated oral gavage on the health of male CD-1 mice. Lab Anim (NY) 2012;41(5):129-34

77. Oliveira PA, MJ Pires, C Nóbrega, et al. Technical Report: Technique of Bladder Catheterization in Female Mice and Rats for Intravesical Instillation in Models of Bladder Cancer. Scand J Lab Anim Sci 2009;36(1):5-9

78. Desesso JM. Confounding factors in direct bladder exposure studies. Comp Toxicol 1989;317-334

79. Cohen SM, Lawson TA. Rodent bladder tumors do not always predict for humans. Cancer Lett 1995;93(1):9-16

80. Vasconcelos-Nóbrega C, Colaço A, Lopes C, Oliveira PA. Review: BBN as an urothelial carcinogen. In Vivo 2012;26(4):727-39

81. Palmeira C, Oliveira PA, Lameiras C, et al. Biological similarities between murine chemical-induced and natural human bladder carcinogenesis. Oncol Lett 2010;1(2):373-77

82. Gil da Costa RM, Oliveira PA, Vasconcelos-Nóbrega C, et al. Altered expression of CKs 14/20 is an early event in a rat model of multistep bladder carcinogenesis. Int J Exp Pathol 2015;96(5):319-25

83. Arentsen HC, Hendricksen K, Oosterwijk E, Witjes JA. Experimental rat bladder urothelial cell carcinoma models. World J Urol 2009;27:313-17

84. Günther JH, Jurczok A, Wulf T, et al. Optimizing syngeneic orthotopic murine bladder cancer (MB49). Cancer Research 1999;59,2834-37

85. Chan ESY, Patel AR, Smith AK, et al. Optimizing Orthotopic Bladder Tumor Implantation in a Syngeneic Mouse Model. The Journal of Urology 2009;183(6):2926-31

86. Levett D, Flecknell PA, Rudland PS, et al. Transfection of S100A4 produces metastatic variants of an orthotopic model of bladder cancer. Am J Pathol 2002;160(2):693-700

87. Ibrahiem EH, Nigam VN, Brailovsky CA, et al. Orthotopic implantation of primary N-[4-(5-Nitro-2-furyl)-2-thiazolyl] formamide-induced bladder cancer in bladder submucosa: an animal model for bladder cancer study. Cancer Res 1983; 43:617-22

88. Bisson JF, Christophe M, Padilla-Ybarra JJ, et al. Determination of the maximal tumor: normal bladder ratio after i.p. or bladder administration of 5-aminolevulinic acid

in Fischer 344 rats by fluorescence spectroscopy in situ. Anticancer Drugs 2002;13: 851-7

89. Chan E, Patel A, Heston W, Larchian W. Mouse orthotopic models for bladder cancer research. BJU Int 2009;104:1286-1291

90. Reis LO, Fávaro WJ, Ferreira U, et al. Evolution on experimental animal model for upper urothelium carcinogenesis. World J Urol 2010;28:499-5

91. Zhang ZT, Pak J, Shapiro E, et al. Urothelium-specific Expression of an Oncogene in Transgenic Mice Induced the Formation of Carcinoma in Situ and Invasive Transitional Cell Carcinoma. Cancer Res 1999;59(14):3512-17

92. Wu XR. Biology of urothelial tumorigenesis: insights from genetically engineered mice. Cancer and Metastasis Reviews 2009;28(3-4):281-90

93. Gollapudi BB, Stott WT, Yano BL, Bus JS. Mode of action considerations in the use of transgenic animals for mutagenicity and carcinogenicity evaluations. Toxicol Lett 1998;102-103:479-84

94. Madka V, Mohammed A, Li Q, et al. TP53 modulating agent, CP-31398 enhances antitumor effects of ODC inhibitor in mouse model of urinary bladder transitional cell carcinoma. Am J Cancer Res 2015;5:3030-41

95. Ching CB, Hansel DE. Expanding therapeutic targets in bladder cancer: the PI3K/Akt/mTOR pathway. Laboratory Investigation 2010;90:1406-14

96. Netto GJ. Molecular biomarkers in urothelial carcinoma of the bladder: are we there yet? Nat Rev Urol 2012;9:41-51

••This paper describes molecular biomarkers in urothelial carcinoma.

97. Foth M, Ahmad I, van Rhijn BW, et al. Fibroblast growth factor receptor 3 activation plays a causative role in urothelial cancer pathogenesis in cooperation with Pten loss in mice. J Pathol 2014;233(2):148-58

98. Mitra AP, Cote RJ, Path FRC. Searching for novel therapeutics and targets: Insights from clinical trials. Urologic Oncology: Seminars and Original Investigations 2007;25: 341-43

99. Bagley RG, Rouleau C, Weber W, et al. Tumor endothelial marker 7 (TEM-7): A novel target for antiangiogenic therapy. Microvascular Research 2001;82:253-62

100. Shin K, Lim A, Odegaard JI, et al. Cellular origin of bladder neoplasia and tisue dynamics of its progression to invasive carcinoma. Nat Cell Biol 2014;16:469-78

101. Van Batavia J, Yamany T, Molotkov A, et al. Bladder cancers arise from distinct urothelial sub-populations. Nat Cell Biol 2014;16:982-91

102. Oliveira PA, Arantes-Rodrigues R, Sousa-Diniz C, et al. The effects of sirolimus on urothelial lesions chemically induced in ICR mice by BBN. Anticancer Res 2009;29(8):3221-26

103. Vasconcelos-Nóbrega C, Colaço A, Santos L, et al. Experimental study of the anticancer effect of gemcitabine combined with sirolimus on chemically induced urothelial lesions. Anticancer Res 2011;31(5):1637-42

104. Arantes-Rodrigues R, Pinto-Leite R, Ferreira R, et al. Meloxicam in the treatment of in vitro and in vivo models of urinary bladder cancer. Biomed Pharmacother 2013;67(4):277-84

105. Ma WW, Adjei AA. Novel Agents on the Horizon for Cancer Therapy. Ca Cancer J Clin 2009;59:111-37

106. Stephenson JJ, Gregory C, Burris H, et al. An Open-Label Clinical Trial Evaluating Safety and Pharmacokinetics of Two Dosing Schedules of Panitumumab in Patients with Solid Tumors. Clinical Colorectal Cancer 2009;8(1):29-37

107. Masters JR, Thomson JA, Daly-Burnsa B, et al. Short tandem repeat profiling provides an international reference standard for human cell lines. PNAS 2001;98(14):8012-17

108. Wallerand H, Bernhard JC, Culine S, et al. Targeted therapies in non-muscle-invasive bladder cancer according to the signaling pathways. Urol Oncol 2011;29(1):4-11

109. Schally AV, Engel JB, Emons G, et al. Use of analogs of peptide hormones conjugated to cytotoxic radicals for chemotherapy targeted to receptors on tumors. Curr Drug Deliv 2011;8(1):11-25

110. Bai J, Tu TY, Kim C, et al. Identification of drugs as single agents or in combination to prevent carcinoma dissemination in a microfluidic 3D environment. Oncotarget 2015;6:36603-14

• This paper describes the microfluidic 3D environment to study in vitro carcinoma dissemination.

111. Fu CL, Odegaard JI, Herbert DR, Hsieh MH. A novel mouse model of Schistosoma haematobium egg-induced immunopathology. PLoS Pathog 2012;8(3):e1002605

112. Kreamer KM. Immune checkpoint blockade: a new paradigm in treating advanced cancer. J Adv Pract Oncol 2014;5:418-31

113. Bracarda S, Altavilla A, Hamzaj A, et al. Immunologic checkpoints blockade in renal cell, prostate, and urothelial malignancies. Semin Oncol 2015;42:495-505

114. Mangsbo SM, Sandin LC, Anger K, et al. Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. J Immunother 2010;33:225-35