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Animal Models for Basic and Preclinical Research in Bladder Cancer

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1. Introduction

Bladder cancer is one of the most common cancers in the world. In 2006, there were about 61,240 diagnosed cases of bladder cancer and approximately 13,060 deaths attributable to this disease, being the prevalence estimated worldwide more than 1,000,000 patients (Jemal *et al.*, 2006; Lerner, 2005). Taking into account that its incidence seems to be increasing, bladder cancer is clearly a significant public health issue around the world. Thus, it is necessary to intensify research on this topic.

Urinary bladder cancer originates mainly from epithelial cells of the urothelium (Lopez-Beltran *et al.*, 2004; Montironi *et al.*, 2005). When initially diagnosed, most bladder cancers (about 70%) do not present muscle invasion, and are thus known as non-muscle invasive bladder cancer (pTa and pT1). In these cases, a simple transurethral resection is sufficient to remove the tumor. However, some patients experience recurrence or even tumor progression. The progression of the tumor involves invasion of tumor cells, which penetrate deeper layers of the bladder such as the detrusor muscle (pT2), perivesical tissue (pT3) and extravesical organs (pT4) (Figure 1). Since this progression threatens the patient's life, more aggressive therapies are necessary (Sobin *et al.*, 1997).

Intensive research in bladder cancer, as well as that in most tumors, is being carried out to elucidate the reason for the appearance of tumors, and to find out which factors are involved in their development and which are related to the tumor progression process. These investigations, which provide insights into the biology of the tumor, are essential for the implementation of new therapeutic and/or preventive modalities (Bhattacharya *et al.*, 2010; Zhang *et al.*, 2011).

Research on basic science is focused on the mechanisms that lead cells towards transformation and development of cancer, using simple experimental models where it is easier to interpret the results. Cell culture techniques are widely used to study different oncological processes. The cell culture is the growth of any cell type, usually tumor cells, in with nutrient-containing solutions. The cells grow attached to the plastic surface, forming a monolayer, usually in a two-dimensional way. This technique allows studying processes such as mutagenesis, invasion, migration, and production of proteolytic enzymes. Although cell culture is a very important tool, it has certain limitations. Many biological processes depend on the three-dimensional architecture. In addition, monolayer culture is usually

restricted to a single or at most two cell types. In contrast, tumors are complex and consist of tumor cells and other cell types such as stroma and immune cells that interact to either promote or inhibit tumor growth. To overcome these limitations, it is necessary to use three-dimensional models, such as tissue or organ cultures (Varley *et al.*, 2011).

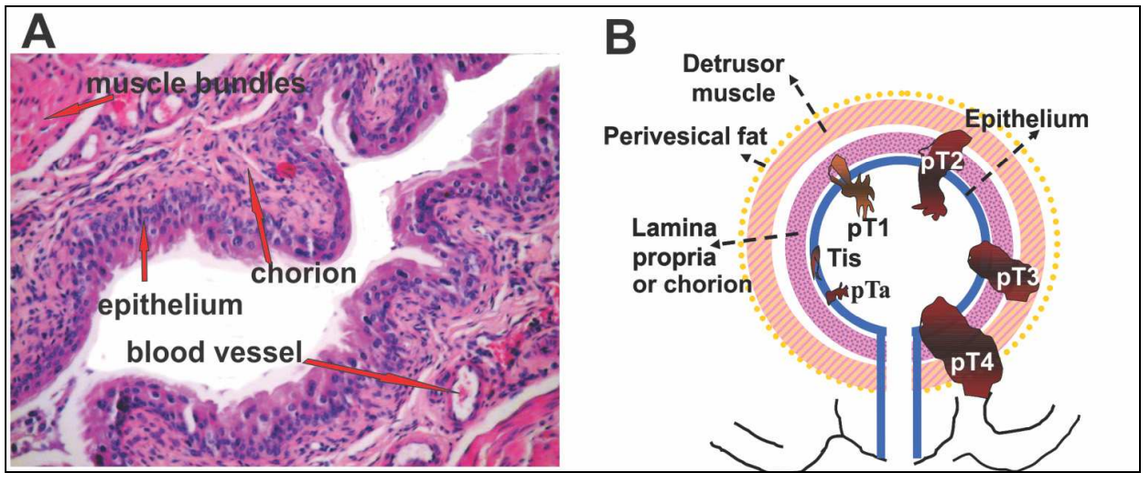

Fig. 1. A: Histology of a normal bladder from a C57BL/6J mouse (Hematoxylin- eosin).
B: Scheme of the invasion status of bladder tumors. Non-muscle invasive tumors pTa when are confined to epithelium, and pT1 when penetrating into the chorion. Invasive tumors when penetrate deeper layers of the bladder such as the detrusor muscle (pT2), perivesical tissue (T3) and extravesical organs (pT4).

To corroborate in vitro results, the next step in the investigation is the assay in a living organism. Animal models are important tools which allow studying the mechanisms of carcinogenesis as well as carrying out preclinical studies of new therapeutic modalities. It is important to design a model as similar to human disease as possible, so that observations can be readily transferred to clinical studies.

2. General characteristics of animal models

Animal models constitute the essential link between cell-based experiments and the translation of novel agents into human patients with cancer. They are used to study the development and progression of diseases and to test new treatments before they are provided to humans. Therefore, models should be as close to human pathology as possible.
In evolutionary biological terms, large animals have more similarity to humans. However, the most widely used animal models are rodents, in particular mice and rats. Although imperfect in their translatability into clinical efficacy, these animals have the advantage that they reproduce easily in short time, are easy to maintain with low cost, and can be manipulated genetically, thus remaining a critical tool in bladder cancer research.
Models allow researchers to study different characteristics of the tumor biology such as tumor growth, latency, growth rate, invasion and metastasis. Studies of carcinogenic substances or prevention of carcinogenesis may be carried out in animal models. Also, analysis of the response to cytotoxicity and immunotherapy treatments can be performed (Bhattacharya *et al.*, 2010; Takeuchi *et al.*, 2011; Zhang *et al.*, 2011).

According to the site of tumor inoculation, models are classified as heterotopic or orthotopic (see points 2.1.4 and 2.1.5.). In addition, depending on the species in which the tumor cell lines are inoculated, models may be xenogeneic or syngeneic models (see below).

2.1 Mouse models in bladder cancer

2.1.1 Xenogeneic models

Animals with transplanted human cancers are called xenogeneic or xenograft models. Nude mice are commonly used to inoculate human tumor fragments or bladder cancer cells. These mice have a spontaneous mutation in chromosome 11 named *nude* (*nu*), which gives certain phenotypic and functional changes. Homozygous nude mice show absence of hair, the feature that gave the name to the mutation. However, a few years after the appearance of the mutation, it was found that nude mice do not have a functional thymus. As a consequence, these animals have a low number of mature T lymphocytes, which allows them to accept xenograft transplantation. This feature of nude mice has contributed to the development of research in cancer, making these animal models useful to study the in vivo growth of human tumors and human cancer cell lines in which the efficacy of therapeutic agents such as monoclonal antibodies, cytotoxic drugs and radiotherapy can be tested. Below are a few examples of the relevant experiments in bladder cancer therapy using xenograft models.

One of the main features of tumors is their capacity to grow uncontrollably, invading the surrounding tissue, inducing neoformation of blood (angiogenesis) and lymphatic vessels and spreading in the body, forming secondary tumors or metastasis. In most cases, the death of patients with bladder cancer is due to the generation of metastasis. Angiogenesis, which is intricately involved in growth and metastasis and is in fact a prerequisite for these processes (Fidler, 1990; Folkman, 1986), is regulated by a fine balance between stimulatory and inhibitory factors produced by the tumor and the surrounding stroma (Liotta *et al.*, 1991). Bladder tumors produce high levels of several stimulatory factors, being the vascular endothelial growth factor (VEGF) overexpressed in bladder cancer (Crew *et al.*, 1997; O'Brien *et al.*, 1995). The action of this factor is mediated by its membrane receptor (VEGFR). Both VEGF and VEGFR are considered as important therapeutic targets. Some papers have studied the effects of a neutralizing monoclonal antibody targeted at murine VEGFR by using a xenograft model. In combination with cytotoxic compounds, such as paclitaxel, this monoclonal antibody impairs tumor growth and angiogenesis and. thus prevents metastatic spread and prolongs mouse survival (Davis *et al.*, 2004; Inoue *et al.*, 2000).

Xenograft models have also been used in radiopharmaceutical studies (Pfost *et al.*, 2009). Pfost et al. coupled monoclonal antibodies that recognize epidermal growth factor receptors on bladder cancer cells with ^{213}Bi, a radioactive alpha particle emitter, and found that therapy with 0.37 MBq of radiation after tumor cell inoculation in the bladder of nude mice results in higher survival of mice when compared with conventional treatment with Mitomycin C. These authors were also able to show that Mitomycin C produces nephrotoxicity, whereas ^{213}Bi-anti-EGFR-mAb treatment showed no signs of nephrotoxicity. These results suggest that radioimmunotherapy using intravesically instilled ^{213}Bi-anti-EGFR-mAb is a promising option for the treatment of bladder cancer in patients.

Xenogeneic models have also been used for the detection of growth and metastasis spread by bioluminescence techniques (Hadaschik *et al.*, 2007). To monitor tumor growth and therapeutic efficacy, noninvasive imaging concepts are preferable. For that purpose, tumor

cells are stably transfected with genes coding for fluorescent proteins (Tanaka *et al.*, 2003) or enzymes catalyzing bioluminescence (Hadaschik *et al.*, 2007), allowing for the continuous visualization of tumor development after intravesical instillation of tumor cells.
Although, as described above, xenograft models are important tools to study the behavior of human tumors in vivo, they also have an important limitation: they are immunodeficient. This makes this animal model not suitable to study interactions between the host immune system and the tumor. Furthermore, xenograft models are useless for research on the biological mechanisms related to carcinogenesis or on the possible compounds able to prevent carcinogenesis. In contrast, syngeneic animal models are more appropriate to approach these issues.

2.1.2 Syngeneic models

Syngeneic models include the appearance of spontaneous tumors, induction of tumors by chemical carcinogens, and inoculation of tumor cells in mice genetically identical to those in which tumors were developed. All of them are useful for studies in which the host-tumor interaction must be taken into account.

Immunotherapy

In patients, non-muscle invasive bladder cancers are usually managed with transurethral resection followed by the intravesical administration of Bacillus Calmette-Guerin (BCG). This immune therapy has been used without modification since 1976 (Morales *et al.*, 1976). In addition to the direct anti-tumor effect (Sandes *et al.*, 2007), it is widely recognized that intravesical BCG therapy is more potent in preventing tumor recurrence than any other intravesical chemotherapy (Sylvester, 2009). However, about 20% of patients either fail to respond initially or relapse within the first five years of treatment (Smaldone *et al.*, 2009). The exact mechanisms of BCG action have not been completely elucidated yet. However, it is known that BCG generates a local immunological reaction with activation of immune cells as well as secretion of cytokines involving Th1 cell cytotoxicity (Riemensberger *et al.*, 2002). To investigate the immune mechanisms by which BCG prevents bladder tumor recurrence and progression as well as the mechanisms of immune suppression to explain the lack of effectiveness of BCG observed in some patients, animal models with a competent immune system are needed. Syngeneic mouse bladder cancer models have thus been used for this purpose.
Animal models using subcutaneous or orthotopic inoculation of bladder cancer cell lines are being designed to study potential therapies to reverse these immune suppressive mechanisms. Mangsbo et al. have studied a syngeneic model by inoculating MB49 bladder cancer cell lines in the subcutis of C57BL/6J mice. This experimental model closely mimicks human bladder cancer, because MB49 cells express negative regulatory proteins of the immune response (Inman *et al.*, 2007; Nakanishi *et al.*, 2007). Among others, the programmed death ligand 1 (PD-L1) and the cytotoxicity T lymphocyte antigen-4 (CTLA-4) render T regulatory cells (Tregs) that can oppose to BCG immunotherapy. Antibodies able to block PD-L1 and CTLA-4 administered intratumorally improves long-term survival and leads to increased levels of tumor-reactive T cells and decreased numbers of Tregs at the tumor site. Therefore, this experimental model has allowed an approach to the understanding of immune suppression during immune therapy with BCG and represents a new therapeutic option in the treatment of bladder cancer (Mangsbo *et al.*, 2010).

It is known that BCG is neither free of mild or intermediate side effects such as fever and granulomatous prostatitis nor of severe side effects such as pneumonitis, hepatitis and BCG sepsis (DeHaven *et al.*, 1992). To avoid such unfavorable events, it is necessary to develop a more active and less toxic immunotherapeutic agent. A mouse syngeneic model using subcutaneous inoculation of MBT2 bladder cancer cell lines has been used to evaluate the effectiveness of liposomes containing walls from BCG bacteria as immune therapy. With this experimental design, Joraku et al. have demonstrated inhibition of tumor growth with increased immunity. Thus, this non-live bacterial agent may contribute to providing a more active and less toxic tool as a substitute for live BCG in immunotherapy (Joraku *et al.*, 2009).
Besides the study of the immune mechanism, other studies involving the tumor-host interaction have used syngeneic models. For example, in our laboratory, we have evaluated the mechanism of action of BCG using animals inoculated subcutaneously with MB49 bladder cancer cells, and found that macrophages from tumor-bearing mice treated with BCG intratumorally were able to produce soluble factors including fibroblast growth factor-2 (FGF-2), which induces fibroblast proliferation. We also found that in vivo BCG therapy reduces tumor growth with a concomitant increase in collagen deposition and expression of alpha-smooth muscle actin and FGF-2. These results suggest that tissue repair mechanisms similar to healing are involved in BCG immunotherapy of bladder cancer (Lodillinsky *et al.*, 2010).

Carcinogenesis and chemo-prevention

Bladder cancer is a candidate for chemo-prevention intervention for several reasons. In the first place, bladder cancer patients present successive recurrences that must be prevented. Also, in addition to genetic susceptibility, this cancer is closely related to exposure to environmental contaminants, including cigarette smoking, which implies the constant contact of carcinogenetic substances with the urothelium.
Animal models are widely used to select chemical synthesis products, purified natural products or even mixtures of natural products with potential to prevent tumor development, which can then be used in clinical trials. The idea is to use organ-specific animal models to determine which agents are likely to be helpful in preventing specific forms of cancer. These animal models can be obtained by chemical induction, spontaneous occurrence or use of transgenic animals.
To be useful, animal models must meet several characteristics. The model should be of clinical relevance, not only in terms of organ specificity but also in terms of the histology and the genetic abnormalities. Furthermore, premalignant lesions should be developed with genetic and histological features as similar as possible to those observed in the development of human cancer. In addition, the model must be consistent in generating tumors in a significant number of animals in a reasonable period. Finally, the model must be predictive in terms of clinical efficacy, i.e. that the positive or negative results obtained in the animal model should later correlate with positive and negative results in human trials (Steele *et al.*, 2010).
One of most useful models is the induction of bladder cancer in mice and rats with hydroxybutyl(butyl)nitrosamine (OH-BBN). This carcinogen compound induces premalignant lesions that progress to transitional bladder tumors, and in little proportion of squamous tumors (Grubbs *et al.*, 2000). Recent studies by Lu et al. have compared bladder tumors in rats and mice induced by OH-BBN with human bladder tumors, using a global gene expression approach cross-species analysis, and shown the similarity between this

animal model and bladder cancer in humans. These genes are likely to have conserved functions contributing to bladder carcinogenesis. To strengthen this analysis, these authors studied the molecular pathway commonly activated in both human and rodent bladder cancer and found a number of pathways that affect the cell cycle, HIF-1 and MYC expression, and regulation of apoptosis in both rodent and human bladder cancer. Also, they compared expression changes at mRNA and protein levels in the rat model and identified several genes/proteins exhibiting concordant changes in human bladder tumors. They concluded that rodent models (in OH-BBN-treated B6D2F1 mice and Fischer-344 rats) of bladder cancer accurately represent the clinical situation to an extent that will allow successful miming of target genes, showing that these models are powerful tools for chemoprevention research (Lu *et al.*, 2010). Using this experimental model, it has been demonstrated that NSAIDs (such as indomethacin, naproxen, NO-naproxen, and celecoxib), various EGFR inhibitors, and purified natural compounds (such as tea polyphenols and sulforaphane) have striking efficacy to prevent bladder tumor development (Ding *et al.*, 2010; Grubbs *et al.*, 2000; Lubet *et al.*, 2005; Steele *et al.*, 2009; Yao *et al.*, 2004).

Two disadvantages inherent in these models are the long experimental times (usually periods between 8 to 12 months) and the occupational exposure of workers. To avoid the use of carcinogens, knockout or transgenic mouse models can be used. These models are used in chemoprevention trials as well as in studies on the relevance of each gene in tumor development.

2.1.3 Transgenic models

Activation of oncogenes or inactivation of tumor suppressors in the urothelium is considered critical for the development of urothelial cancer. Transgenic mice have proven to be powerful tools to unravel the mechanisms of carcinogenesis and to understand the molecular basis of the disease. Transgenic mice are a particular case of syngeneic models, which are genetically modified to study the importance of a particular gene in cancer development and progression. Knockout mice, which are genetically modified mice, can be used to study the effect on the deficiency of a particular gene.

Alterations in the suppressor genes RB1 and p53 as well as the activation of oncogenes such as Ha-ras are commonly found in human urothelial tumors. Transgenic mice with alterations in these genes have been designed. By way of example, we will next describe some of the models developed and the conclusions that have been reached.

Mouse embryos lacking the retinoblastoma (Rb) gene die 14 days into gestation and mice lacking the p53 gene succumb to thymic lymphomas at seven months of age. So, the role of these genes in the analysis of tumorigenesis was delayed until conditional transgenic mice were developed. These models achieve the loss of gene function only in a particular tissue. The specific urothelium knockout system was developed using the Cre/loxP strategy. Transgenic mouse lines in which a 3.6-kb mouse uroplakin II promoter is used to drive the expression of Cre recombinase (Cre) have been generated (Mo *et al.*, 2005). The use of this model has allowed understanding the role of antitumor genes such as RB, p53 and PTEN in bladder carcinogenesis (Ahmad *et al.*, 2011; Ayala de la Pena *et al.*, 2011; He *et al.*, 2009). Conditional inactivation of both RB1 alleles in the mouse urothelium instead of accelerate urothelial proliferation, profoundly activated the p53 pathway, leading to extensive apoptosis in urothelial cells. Thus, pRb loss triggers fail-safe mechanisms whereby urothelial cells can evade tumorigenesis. Additional loss of p53 in pRb-deficient urothelial cells can

remove these p53-dependent tumor barriers, resulting in hyperplasia or umbrella cell nuclear atypia. Also, superficial papillary bladder tumors of low-grade (rare occurrence) but not invasive carcinomas have been detected. Furthermore, mice deficient in both pRb and p53 are highly susceptible to carcinogen exposure, developing invasive carcinomas that resemble human bladder cancer (He *et al.*, 2009). Another transgenic mouse with inactivation of the tumor suppressor p53 has been obtained by expression of SV40 large T antigen, directed to the urothelium with the specific promoter uroplakin-II. In the same way as in the transgenic mice described above, this construction has allowed demonstrating that the elimination of p53 alone is not sufficient for the generation of bladder tumor (Ayala de la Pena *et al.*, 2011). The function of proto-oncogene activation has been assessed by using Ha-ras trangenic mice (Zhang *et al.*, 2001).

Transgenic mice with compromised immune systems have also been developed. Mice knockout to IFN gamma (IFNɣ -/-), interleukins 17, 12 and 23 (IL-17 -/-; IL-12-/- and IL-23 -/-), among others, are being used to understand how different components of the immune system either promote or inhibit the development of bladder tumors (Kortylewski et al., 2009; Langowski et al., 2006; Wang *et al.*, 2009).

In both syngeneic and xenogeneic models, tumors can grow in heterotopic or orthotopic sites. Below we describe the advantages and disadvantages of both modalities.

2.1.4 Heterotopic tumor growth

This site of inoculation refers to the growth of a tumor in a site different from its target organs, generally using subcutaneous inoculation. This approach is advantageous in cases where the orthotopic inoculation (see below) is complex such as in bladder, kidney, and bowels. Tumor inoculation is simple and can be carried out by an operator with minimum training. Furthermore, the tumor can be easily detected and the tumor evolution can be easily assessed by using palpation of the skin and measurement with a caliper, respectively. To assess tumor growth, at least two perpendicular diameters, the larger diameter (D) and the smaller diameter (d), must be measured. Some researchers also measure depth (Figure 2). However, the latter is difficult to determine and generally produces large errors. Tumor size can be calculated from these data, using various formulas such as geometric mean (**$(Dxd)^{1/2}$** expressed in millimeters), arithmetic average (**Dxd/2** expressed in mm^2), or volume of the ellipsoid (**$4/3\ \pi\ Dxd^2$**, expressed mm^3). Not all tumors grow in the same way; some of them are more compact, whereas others develop necrosis. Therefore, to choose the most appropriate formula for each tumor, it is first necessary to validate the formula that best fits, when compared with tumor weight.

The main disadvantage of the heterotopic model is the fact that an anatomic site other than an orthotopic site can differentially develop tumor growth. The tumorigenesis and metastatic potential of tumors depend not only on the characteristics of the tumor cells, but also on the tumor environment and therefore on the site of injection. Human tumors can be formed by different cell subpopulations with varying ability to metastasize and susceptibility to treatment, depending on the site of inoculation (Fidler, 1986). It has been observed that subcutaneous inoculation of murine MB49 bladder cancer cell lines induces lung metastases, and that inoculation of these cells in the bladder does not (Lodillinsky *et al.*, 2009). Similar observations have been made for human tumors using 253J B-V cells (Black *et al.*, 2007). After 28 days of tumor growth either in the bladder or in the subcutis, Black et al. were able to determine that the tumor size was similar in both sites, but that only

tumors growing orthotopically in the bladder developed metastasis to lymph nodes and lungs. The orthotopic tumors, as compared to the subcutaneous tumors, have an increased microvessel density, increase in growth factors expression and proteolytic enzyme activity. Therefore, models of orthotopic growth are more appropriate for studies related to metastasis dissemination or response to any treatment.

Fig. 2. Heterotopic tumor growth: MB49 bladder cancer cells growing in the subcutis of C57BL/6J mice.

There are some heterotopic models such as inoculation into the tail vein or the left ventricle of the heart which have been widely used to evaluate the process of extravasations and colonization in the lung or bone, respectively (Growcott, 2009; Wu *et al.*, 2010). Although very used, these models consider only a limited aspect of the metastatic process (Figure 3).

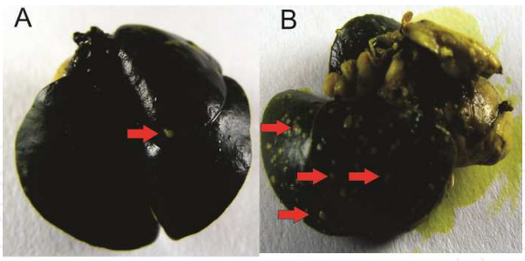

Fig. 3. Lung metastases: inoculation of MB49 (panel A) or MB49-I (panel B) bladder cancer cell lines into the tail vein induces lung metastasis. The lungs are colored in black by intratracheal inoculation of Indian ink. Metastases are seen in yellow by the stain and Bouin's fixative solution.

2.1.5 Orthotopic tumor growth

Growth in the target organ allows for better analysis of the interaction between the host and the tumor. When tumors are chemically induced, the carcinogen is chosen such that the tumor develops in the desired organ. Thus, tumor growth occurs in the orthotopic site. However, in the case that the tumor is generated by inoculation of a tumor fragment or

tumor cell lines, the orthotopic inoculation is not always easy to perform. However, this difficulty must be overcome since the results obtained with heterotopic models are not always easy translated into clinical trials.

As previously mentioned, there are examples showing that different results are observed when the tumor is inoculated subcutaneously or orthotopically in the bladder. In studies of chemoprevention, inhibition of bladder cancer development by allyl isothiocyanate was detected for tumors growing orthotopically but not in the subcutis (Bhattacharya *et al.*, 2010). Furthermore, as described in the previous section, considerable variation has been detected between the two models in assays of immunotherapy, angiogenesis, invasion and metastatic spread, among others. Taking these limitations into account, to achieve a correct interpretation of results and a translatable preclinical model, it is necessary to inoculate the tumor in the bladder.

Inoculation into the bladder requires a qualified technician. Mice must first be anesthetized and subsequently, a 24-gauge Teflon i.v. catheter must be inserted through the urethra into the bladder using an inert lubricant to avoid discomfort in mice. For successful implantation of the bladder tumor cells, the urothelium must first be damaged. There are different techniques to induce such damage in the bladder. One of them involves the use of hydrochloric acid (0.1 ml 0.1 M HCl for 15 minutes) and subsequent neutralization with alkali and extensive washing with saline (Zhang *et al.*, 2011). Another technique involves instillation of a solution of silver nitrate (NO_3Ag) (Chade *et al.*, 2008). Both forms of injury allow the generation of tumors uniformly distributed in the bladder. The inoculation of MB49 tumor cells ($1x10^5$ to $5x10^5$) in syngeneic mice generates superficial tumors in about 7 to 15 days. Other techniques, using polylysine instillation, intramurally inoculation via laparotomy, or electro cauterization of the urothelium, are also used (Black *et al.*, 2010).

Cauterization of the bladder mimics transurethral resection of bladder tumor and therefore should facilitate adherence of instilled tumor cells to the bladder wall. The method was designed by Gunther et al. for the inoculation of MB49 cells in syngeneic mice (Gunther *et al.*, 1999). However, it is also used for inoculation of cells from human bladder tumors in nude mice (Pfost *et al.*, 2009). The technique involves the insertion of a guiding wire into the bladder of a mouse positioned dorsally on the ground plate of the cautery unit via the teflon catheter. When it is verified that the wire touches the bladder wall, the wire is attached to the cautery unit, and a monopolar coagulation mode is applied for 2 seconds at the lowest level (7 W). Then, via the same catheter, an appropriate number of tumor cells are inoculated and should remain in the bladder for at least 30 minutes (Figure 4A).

Another difficulty to be overcome is the determination of the evolution of bladder tumor growth. Unlike what happens in the case of a subcutaneous tumor growth, where its size can be easily determine at different times of evolution, the growth evolution in the bladder is more uncertain. However, hematuria is the hallmark of tumor presence (Figure 4B). Mice with $1x10^4$ or $1x10^5$ MB49 cell lines, inoculated by electrocautery, present hematuria about 15 or 9 days post-inoculation, respectively (Lodillinsky *et al.*, 2009). Inoculation of $5x10^3$ cells in the bladder previously treated with NO_3Ag generates hematuria in all mice about 7 days after tumor implantation (Chade *et al.*, 2008). Palpation of the bladder may give an idea of the extent of the tumor, but it is difficult to carry out because the bladder is retropubic. Also, in some cases, palpation could be given a wrong interpretation. When there is an obstruction of the urethra by blood clots, the bladder is greatly enlarged as a product of the accumulation of urine, and may thus lead to a wrong estimate of the extension of the tumor

(Figure 4C and D). Therefore, in these cases, the true evaluation of tumor size can be obtained at the end of the experiment, either by measuring the bladder with a caliper or by determining its weight (Figure 4E and F). Experiments of this type, also called end-point, have the disadvantage that they focus only on one measure of tumor size and not on its evolution throughout the experiment. This problem will soon be overcome by the design of non-invasive diagnostic equipment for small animals, similar to those used in medical practice in humans, such as ultrasound-doppler, infrared (IR) or bioluminescence imaging. By way of example, ultrasound-Doppler sagittal images have been used to evaluate angiogenesis in a mouse bladder cancer model (Sugano *et al.*, 2011). Also, bladder cancer cells that have been engineered to express certain proteins that emit fluorescence are being used in bioluminescence detection of tumor development (Black *et al.*, 2010).

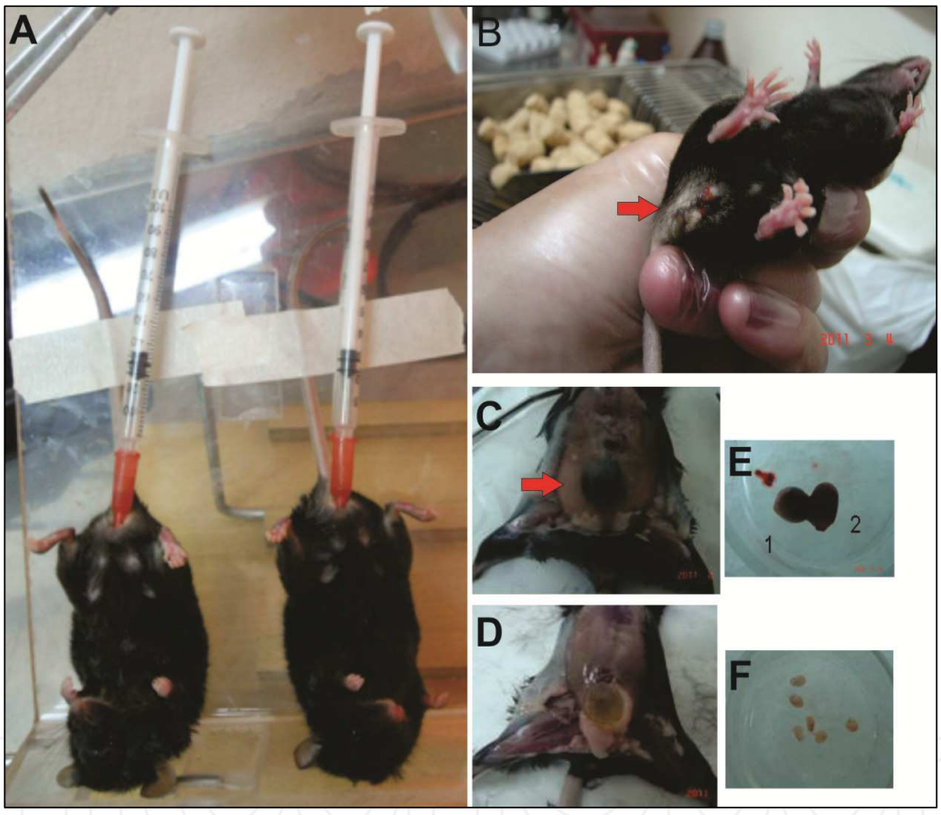

Fig. 4. Bladder tumor growth: A) orthotopic inoculation: after cauterization and inoculation of the appropriate number of tumor cells, mice should remain upside-down for at least 30 minutes so that cells can adhere to the bladder epithelium. B) Hematuria is the hallmark of tumor presence. C) Mouse with bladder tumor. D) Mouse with bladder containing urine but without tumor. E) Two bladders with tumor. F) Bladders from normal mice.

2.2 Mouse bladder cancer model for study of invasion and metastasis

2.2.1 Invasion and metastasis

The process of tumor invasion and metastasis is the most devastating stage of neoplastic disease and worsens prognosis of cancer patients. Adverse effects of systemic anti-tumor therapy and organ failure invaded with metastatic tissue are the leading causes of death in

these patients (Steeg *et al*., 2006). It is currently accepted that tumors have a clonal origin, which means that they are derived from a single cell. The high proliferative capacity, coupled with the genetic instability of tumor cells, generates new mutations, and thus the generation of other cell populations conferring tumor heterogeneity. This is considered part of an evolutionary process of genetic and epigenetic changes that allow some of the primary tumor cells acquire an adaptive advantage to migrate and colonize new environments. However, new findings have shown the possibility of a parallel development of cells capable of early metastatic spread. This parallel progression model urges to review the current diagnostic and treatment (Klein *et al*. 2009).

The local invasion process that gives rise to metastatic spread is a multi-step event called metastatic cascade. This is a phenomenon with low efficiency, indicating that only a few of the cells that emerge from the primary tumor are able to generate metastases.

Initially, tumor cells release proteolytic enzymes, such as MMPs and cathepsins, which degrade the extracellular components of basement membrane, thereby creating gaps that allow the invasion of the underlying connective tissue. The tumor cells migrate through the extracellular matrix and some may penetrate the lymphatic and blood capillaries, a phenomenon knows as intravasation. Once in the bloodstream, cells that manage to survive must leave the vessel (extravasation) into different organs (Figure 5). When the microenvironment of the target organ is appropriate, colonizing tumor cells can form metastases.

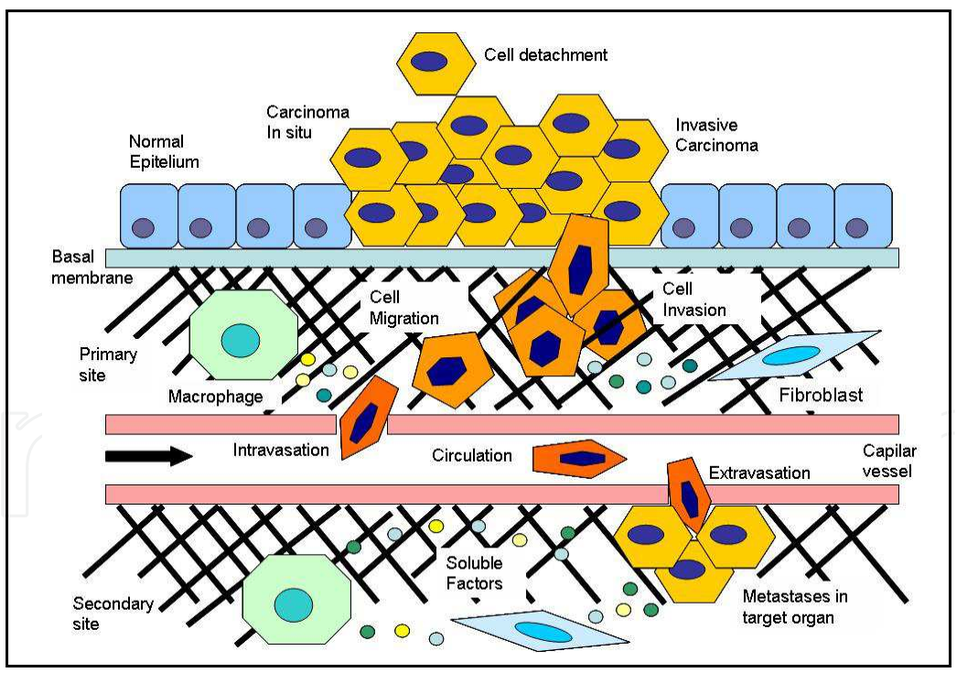

Fig. 5. During the metastatic cascade, tumor cells undergo genotypic and phenotypic changes that increase their capacity for invasion and migration. Some tumor cells are capable of degrading the basal membrane and migrating through connective tissue. Cells from connective tissue, such as macrophages and fibroblasts, through the release of growth factors, cytokines and proteolytic enzymes, can enhance the invasive behavior of tumor cells.

2.2.2 Metastogenes

The study of gene expression in primary tumor cells and metastatic cells has begun to lift the veil that prevented the understanding of the complex process of metastasis. These studies have identified a set of genes involved in the development of metastasis, called "metastogenic genes". Although there may be some overlap, these genes have been classified into three categories: a) initiation genes, b) progression genes and c) virulence genes (Nguyen *et al*., 2007).

Initiation genes are associated with the processes of invasion, angiogenesis and epithelial-mesenchymal transition (EMT). Since during invasion the migration of cells is an important step, genes encoding for GTPases involved in cytoskeleton remodeling such as RhoC have been included in this category. Among those involved in angiogenesis are those encoding the vascular endothelium growth factor, and matrix metalloproteinase 9 (MMP9). EMT allows changes that give advantages in terms of migration and invasion. Certain genes that encode for transcription factors associated with this transition such as TWIST1 are also included in this group.

Progression genes are linked to the negative regulation of the immune response, vascular remodeling and extravasations. Examples are the gene coding for cyclooxygenase 2 (COX-2), matrix metalloproteinase 1 (MMP-1) and angiopoietin-like 4 (ANGPTL4), among others.

Finally, virulence genes are those which give the tumor cell an adaptive advantage to survive within an organ-specific microenvironment (Chiang *et al*., 2008). Among them are intercellular signaling molecules such as cytokines and interleukins (CXCR4 and IL-11), molecules of the family of tumor necrosis factor (TNF) that are associated with bone metabolism (RANKL) and mediators of the angiogenic process such as the Endothelin-1.

Recent findings have identified the expression pattern characteristic of primary tumor gene, which is similar to a genetic signature that predicts the metastatic potential of the tumor (Bertucci *et al*., 2007, Van't Veer *et al*., 2002). This implies that the genetic profile expressed in metastases in specific organs is not always the same. Different groups of genes allow tumor cells to interact with stromal cells of the target organ. For example, the genes involved in breast cancer metastasis to bone are different from those involved in metastasis to the lung. This knowledge would allow the development of therapeutic strategies specific for each gene expression pattern or "signature " of a metastasis.

2.2.3 Epithelial-mesenchymal transition

During the invasion process, the tumor cells show a phenotypic change called epithelial-mesenchymal transition (EMT), which is characterized by a morphological change that is due to a genetic reprogramming process which normally occurs during embryonic development and tissue repair such as scarring (Peinado *et al*., 2007). This reprogramming involves the expression of a group of transcriptional repressors (Zeb-1 and 2, Twist, Snail and Slug) that recruit histone deacetylases, controlling the expression of genes associated with the epithelial phenotype. An example of this is the decreased expression of E-cadherin, which leads to a loss of homotypic adhesion. Certain cytokines of the family of transforming growth factor beta (TGFbeta) and bone morphogenetic protein (BMP) are responsible for increasing the expression of these repressors (McConkey *et al*., 2009).

Simultaneously with an underexpression of proteins of the epithelial phenotype, an overexpression of molecules associated with the mesenchymal phenotype has been detected. The expression of vimentin and loss of apical-basal polarization is a characteristic change of cells undergoing EMT (Peinado *et al*., 2007).

2.2.4 Proteolytic enzymes in bladder cancer invasion

Proteolytic activity is of fundamental importance for the development, growth and maintenance of homeostasis of all the tissues in any organism. In each particular tissue, the activity of proteolytic enzymes is regulated at different levels, both at gene expression, transcriptional regulation and by specific endogenous inhibitors (Durkan *et al.*, 2003; Kumar *et al.*, 2010). In addition, these enzymes can activate each other through a mechanism cascade that also regulates their activity. The genetic instability of tumor cells leads to alterations in the genes encoding proteolytic enzymes and/or their inhibitors, which lead to an increased proteolytic activity in the tumor. It is well documented that proteolytic enzymes are involved in the process of invasion and metastasis. Matrix metalloproteinases (MMPs),cathepsins (B, L) and urokinase-type plasminogen activator (uPA) are the three main groups of enzymes described in the process of tumor invasion.

MMPs, of which several isoforms are known, have a major role in matrix destruction and are involved in metastasis by mediating basement membrane destruction and angiogenesis (Kim *et al.*, 2004). Of all known isoforms, MMP-2 and MMP-9 are strongly associated with invasion in bladder cancer (Eissa *et al.*, 2007; Papathoma *et al.*, 2000).

Cathepsins have also been involved in cancer invasion. Cathepsin B (CB) is one of the most abundant lysosomal cysteine proteinase in mammalian tissue. It is synthesized as a glycosylated zymogen named pro-CB and subsequently converted to an active form of 33 kDa or 27-29 kDa. CB has an important role in the lysosomal degradation of proteins and is also involved in the degradation of the extracellular matrix in neoplastic and inflammatory diseases. Particularly, results from our laboratory have shown that the high expression of the active form of CB in transitional bladder tumors is associated with worse prognosis factors such as invasiveness and high histological grade (Eiján *et al.*, 2003).

The proteolytic activity of uPA is a system regulated by urokinase, its specific receptor uPAR and the specific plasminogen activator inhibitor 1 (PAI-1). This system plays a major role in tumorigenesis, tumor progression, tumor invasion and metastasis formation. It is generally assumed that the pro-malignant effect of the uPA-uPAR system is mediated by increased local proteolysis, thus favoring tumor invasion, as well as by the pro-angiogenic effect (Binder *et al.*, 2008). Consistent with this activity it has been shown, in a rat orthotopic model, that intravesical administration of PAI-1, which inhibits uPA activity in tumors, reduces the growth and progression of bladder cancer (Chen *et al.*, 2009).

2.2.5 Orthotopic mouse bladder cancer invasion model

Certain fundamental properties of metastatic cells such as migration and invasion can be studied in the laboratory using tumor cell cultures. Using various tools of genetic engineering, genes that encode molecules that emit fluorescence (green fluorescent protein), bioluminescent molecules (luciferase) or molecules with color (beta galactosidase) can be introduced into the cell. This technique is known as reporter gene and has allowed the analysis of molecular processes at the level of cell groups or isolated cells (Ghajar *et al.*, 2008; Menon *et al.*, 2009). In vitro experiments have also been useful to shed light on genes that might be involved in certain steps of the metastatic cascade. So, the use of genetic and pharmacological methods has shown that the expression of certain genes facilitate the assembly of new tumor blood vessels, tumor cells out of circulation and the passage of circulating tumor cells through the pulmonary capillaries to grow lung metastases (Gupta *et al.*, 2007; Valastyan *et al.*, 2009). However, in vitro models allow a simple analysis and do not

always allow evaluating interactions with the tumor microenvironment. It is therefore important to develop animal models to analyze the factors associated with tumor progression (Bos *et al.*, 2010). To this end, we have added the advances in multiphoton intravital microscopy, which allows observing the in vivo behavior of tumor cells labeled with green fluorescent protein in the process of invasion and metastasis (Condeelis *et al.*, 2003).

There are only few useful animal models to study the processes of invasion and metastasis. Dinney et al. have designed an orthotopic murine model with different degrees of invasion. To this end, they seeded human 253J cells into the bladder wall of immunodeficient mice (nude) and then selected subpopulations of the parental line by in vivo reimplantation in the bladder. After five serial passages, tumors were more tumorigenic and showed metastatic capacity (Dinney *et al.*, 1995). These authors observed that these variants had a tumoral abnormal karyotype, increased expression of molecules such as epidermal growth factor receptor (EGFR), interleukin 8 (IL-8) and MMP-9, and also observed an increased anchorage-independent growth and increased capacity to migrate in Matrigel ® (trade name of a protein mixture secreted by mouse sarcoma cells Engelbreth-Holm-Swarm), commonly used in the study of invasive and migratory behavior of tumor cells in contact with extracellular matrix components.

This is a xenogeneic model in which it is possible to study the changes experienced by the tumor cells to acquire their invasive and metastatic phenotype. While this is an ingenious and very useful model, the fact that it is an immunodeficient mouse slightly restricts the applicability of the model.

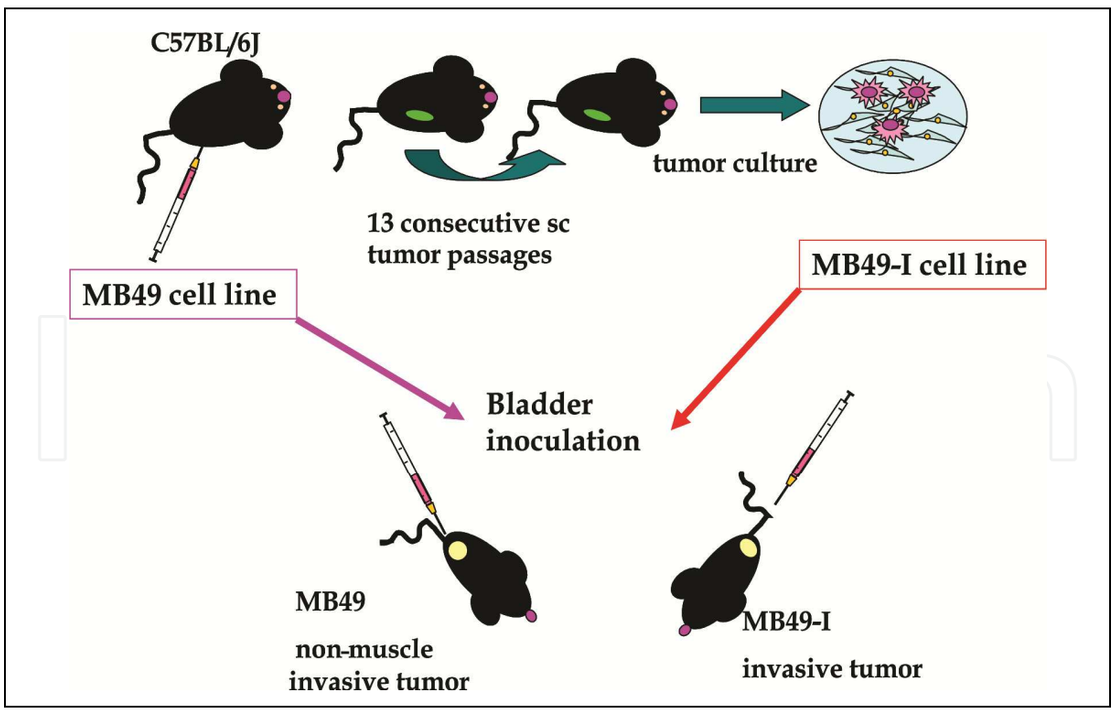

Fig. 6. MB49-I cell line obtained by successive in vivo passages of primary tumor obtained by inoculation of the MB49 bladder tumor cell line. The orthotopic inoculation in bladder of MB49 or MB49-I generates non-muscle invasive or muscle invasive tumors respectively.

In our laboratory, following the methodology of Dinney, but with subcutaneous inoculation, we developed a syngeneic murine model that reproduces the human pathology in terms of invasion status. A single cell suspension of the MB49 cell line was inoculated subcutaneously in the flank of syngeneic C57BL/6J mice. After 24 days, tumors were surgically removed and 2-mm tumor pieces were transplanted by trocar into the left flank of mice. This process was repeated 13 consecutive times. We found that the growth rate was increased in transplants #6 to #10 and then became stable. Therefore, primary culture from transplant #13 was carried out and the cell line originated was named MB49-I. The orthotopic inoculation of MB49 or MB49-I in the bladder generates non-muscle invasive or muscle invasive tumors respectively (Figure 6).

This new line has more aggressive characteristics. The MB49-I cell line has higher activity of the MMP-9, uPA and CB as well as increased in vitro invasion of Matrigel ®. Given the association of these enzymes with bladder cancer progression, our model has close similarities to human disease. The histopathological study in vivo showed results consistent with in vivo tests. Intravesical inoculation of MB49 cells was able to develop tumors without muscle invasion. By contrast, inoculation of the MB49-I cell line generated carcinoma with a disorganized structure and larger tumors with cellular atypia, muscle layer invasion and lung metastasis (Figure 7).

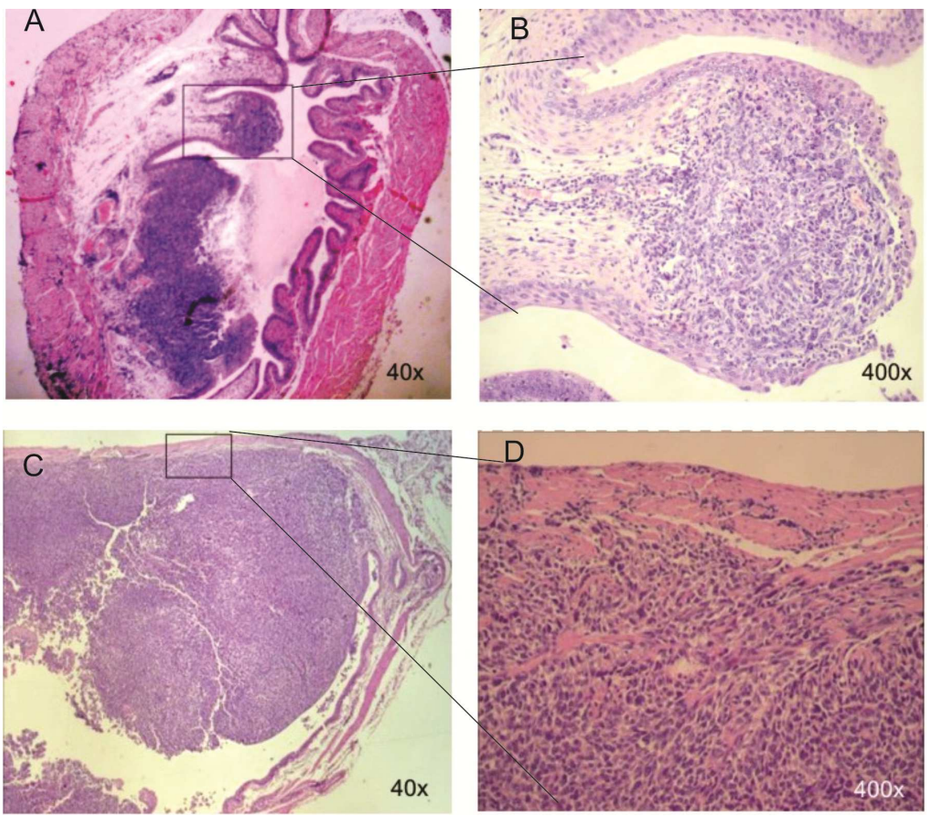

Fig. 7. Haematoxylin-eosin staining for bladder tumors. A: It can see two formations of a tumor of bladder MB49: one sessile and one pedunculated, which do not invade the muscle wall. B: MB49 the polypoid formation is magnified 400X. C: image of a tumor MB49-I, we observe a large tumor invading the muscular layer. D: MB49-I bladder tumor with higher magnification, where the tumor cells are intermingled with the muscle fibers.

Cellular plasticity is a fundamental process during tumor progression. It is now accepted that epithelial-mesenchymal transition is associated with tumor progression. A mark of this transition is the loss of cytokeratin and an increase in vimentin. MB49-I has not only morphological diversity, but also decreased cytokeratin and increased vimentin expression in vitro and in vivo.
Since both the xenogeneic and syngeneic models described here resemble human bladder cancer, they could be useful to study tumor progression, tissue remodeling, and invasive and metastatic processes, and to assay anti-invasive and metastatic agents.

3. Conclusions

Since animal models can reproduce the tumor-host interactions, performing studies using these models is a mandatory step to translate from basic research to the clinic. Taking into account that animal experiments are performed to obtain an improvement for human-health, but must generate a reduced impact on the animals, these experiments should be made according to international rules of bioethics. To accomplish the maximum welfare of the animal, every protocol, indicating the justification of each experiment and the methodology to be used, must be approved by the institutional ethics committee for the use of laboratory animals.
The ideal animal model should meet all the characteristics of the human pathology, such as growth parameters, histology, evolution and metastatic dissemination. However, in the practice, the ideal model does not always have a complete similarity. The researcher must thus decide which model best fits the question to be answered. Alternatively, the researcher can design his/her own model that most closely approaches the point of interest.
The generation of transgenic animals is one of the most developed branches in animal models. Technical refinements have allowed generating genetically modified mice either stable or conditional, making them a valuable tool.
Animal models that mimic human bladder cancer in terms of invasion have also been developed. The use of successive transplants of tumors derived from a cancer cell line can generate invasive bladder tumors. Both the syngeneic and xenogenic invasive models of tumor are useful in the study of tumor progression. Finally, it is important to note that for a better understanding of the tumor mechanism and the relationship with the host, the best models are those, like MB49-I, in which the tumor is inoculated in an orthotopic site.

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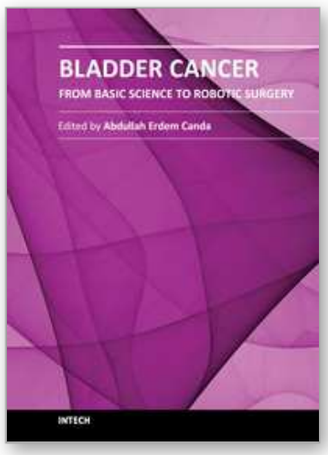

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This book is an invaluable source of knowledge on bladder cancer biology, epidemiology, biomarkers, prognostic factors, and clinical presentation and diagnosis. It is also rich with plenty of up-to-date information, in a well-organized and easy to use format, focusing on the treatment of bladder cancer including surgery, chemotherapy, radiation therapy, immunotherapy, and vaccine therapy. These chapters, written by the experts in their fields, include many interesting, demonstrative and colorful pictures, figures, illustrations and tables. Due to its practicality, this book is recommended reading to anyone interested in bladder cancer.

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