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Review

Experimental Models for Therapeutic Studies of Transitional Cell Carcinoma

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Abstract. *Transitional cell carcinoma (TCC) is the second most common malignancy of the genito-urinary tract. To date, the therapy options available seem unable to prevent TCC recurrence and its progression, even though therapy studies are constantly investigating new treatment options. A great variety of TCC models have been described. For each study, the model has to be modified according to the specific investigative aim. The establishment of reproducible models is a continuing challenge. Currently, there is a strong tendency towards 3-dimensional models for in vitro studies. The aim of this article was to review current ex vivo and in vivo models used in studies of TCC therapy. We describe 2- and 3-dimensional cell and tissue cultures, as well as the set-up, investigative options and detection methods used in rodent heterotopic and orthotopic models.*

In 2006, it was estimated that there were more than 105,000 new cases of bladder cancer and over 36,000 deaths from the disease in Europe (1). The most common type (>90%) of bladder cancer in Europe is transitional cell carcinoma (TCC), which occurs in the lower and upper urinary tract. It originates in the urothelium and can progress *via* invasion of the lamina propria and the detrousor muscle to a metastatic disease. Squamous cell carcinoma and adenocarcinoma are rare types of bladder cancer, constituting about 2.5% and 2%, respectively, of the disease (2).

In bladder TCC, 70-80% of the tumours are confined to the urothelium (*i.e.* Ta, Tis, T1). At these superficial stages, local medical treatment can be instilled directly into the bladder *via* the urethra. The current gold standard for treating superficial TCC with a low risk of progression is transurethral resection and intravesical chemotherapy with different agents, *e.g.* Mitomycin C (3). However, to date no effective method of preventing recurrence has been found. Recurrence of superficial TCC occurs in up to 70% of all cases, 10-15% of which progress to an invasive disease (4).

For high-grade tumours and carcinoma *in situ* maintenance therapy with a chemotherapeutic agent or Bacillus Calmette-Guérin (BCG) is indicated. Intravesical application of BCG has been found to reduce the overall risk of recurrence and progression, but the response rate is only 60-70% (4). Even though prevention is becoming more and more of an issue, non-invasive screening methods for diagnosing bladder TCC early are still not sufficiently developed for routine clinical use. No effective chemoprevention can be applied. Therapy remains the main option for combatting bladder TCC, but is still insufficient in the cases of most patients. Representative experimental models are therefore needed so that more effective treatments can be developed in preclinical evaluation. A great variety of *ex vivo* and *in vivo* models has been described in the literature. This article reviews the current *ex vivo* and *in vivo* models for TCC with regard to design and implementation, their usefulness for different investigative aims, detection methods and the evaluation of results from experiments.

Ex Vivo Models

Cell culture.

Human TCC cell lines. The first human bladder cancer cell line, HeLa, was described in 1952 (5). Today, cell cultures are used in different scientific areas: in research aimed at understanding cell functions, in screening processes for detecting active compounds and in the production of biopharmaceuticals. In urological research, human urothelial cell lines are well-established tools for preclinical trials. A wide range of cell lines is provided by cell banks and originators, derived from different TCCs such as primary or

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Table I. *Origin of human TCC cell lines.*

Cell line	Origin of biopsy	Clinical stage of disease	Established in	Gender	Reference
5637	Primary TCC	n.a.	1974	Male	(79)
BC-3C	Invasive, solid TCC	G4, C	1998	Female	(80)
CAL-29	Invasive, metastatic TCC	G4, T2	1985	Female	(81)
HT-1376	Invasive TCC	T2, G3	1977	Female	(82)
HT-1197	Recurrent invasive TCC	T2, G4	1977	Male	(82)
KU-19-19	Invasive TCC	pT3b, G3	1993	Male	(83)
RT4	Recurrent TCC	G1, T2	1968	Male	(6)
RT112/84	Primary TCC	G2	1973	Female	(84)
T24	Primary TCC	G3	1970	Female	(85)
U-BLC1	Primary TCC spontaneously immortal	min. pT2, G3	1998	Female	(86)
UM-UC 3	TCC	n.a.	1982	Male	(87)

n.a.: not applicable.

recurrent disease as well as from the normal urothelium (6). Depending on their origin, they differ in stage and grade. To date, many cell lines are well characterised in their histology and tumourigenicity (Table I).

Superficial bladder tumours consist of heterogeneous cell populations (7, 8). Therefore, to be a representative model, a cell line ought to present a similar cell composition. Potentially, uncloned cell lines still present the required genetic and phenotypic biodiversity, even though they can be considered abnormal simply by having undergone the immortalisation process.

Attention has to be paid to the origin of cell lines to ensure that they are reliable, because cross-contaminated sub-lines occur frequently. Cross-contamination is created when a cell line was accidentally coinfected or replaced by a different line, but maintains its primary name because the change went unnoticed. This provides misleading research results and might lead to false statements. Masters and co-workers found a total of nine cell lines cross-contaminated with T24, a line started from bladder TCC in 1970 (9). Four of the tested cell lines were 100% identical to T24, but traded under a different name. Cross-contamination can be detected in several ways, including short tandem repeat profiling and enzyme polymorphism (9, 10).

To avoid mistakes, cell lines should be purchased from the originator or from authenticated cell banks, *e.g.* the European Collection of Cell Cultures (www.ecacc.org.uk), the German Collection of Microorganisms and Cell Cultures (www.dsmz.de), or the American Type Culture Collection, Virginia (www.atcc.org).

Cell lines in culture. Cell lines are grown under standardised conditions according to the originator's instructions. Commercial culture media with different supplements, *e.g.* fetal bovine serum, amino acids and salts, are available. Bacterial infection can destroy the cultures and must be prevented by ensuring aseptic working conditions and using antibiotic in the culture medium. The use of non-standardised mediums may adversely affect the quality and reproducibility of a culture. For proliferation, cells are usually incubated under standard conditions at 37˚C and 5% CO_2. When cell populations reach a sub-confluent stage they are passaged on.

Cells grow in monolayers or multilayers, depending on the underlying surface. When cultured in plastic cell culture flasks, permanent cell lines proliferate as two-dimensional monolayers. If seeded on an extracellular matrix (ECM), they form three-dimensional multilayers. Different organic tissues and artificial ECMs are widely described in the literature. Cells can either be seeded onto membranes, foams, sponges and organic tissue, which are sometimes pretreated or coated, or be embedded into collaginous gels to induce a three-dimensional structure (11-15). The use of ECMs will improve cell differentiation and adhesion. The culture medium, nutrition schedule and composition of the ECM have an immense influence on cell properties, gene expression and phenotype, to name but two (16-18). A precise protocol must therefore be followed if reproducibility is to be ensured. However, these parameters might also be helpful in optimising cultures.

Kyker and co-workers examined the behaviour of TCC cell lines according to differences in culture conditions (16). When fed twice weekly with medium containing 10% fetal calf serum (FCS), the TCCsup and J82 lines grew as an invasive structure in keeping with their aggressive origin on a small intestine submucosa (SIS) gel. When fed daily under the same conditions, the cells formed a layered, non-invasive structure. The RT4 line grew in a normal layer when fed with 10% FCS medium, independent of the feeding schedule, whereas a papillary structure occurred in 1% FCS medium.

A similar study compared the phenotype of five TCC cell lines grown on Matrigel, a cancer-derived ECM, and SISgel, an ECM that is derived normally (17). On Matrigel, the invasive cancer cell lines grew invasively and the low-grade lines developed papillary structures. On SISgel they grew as layers and were non-invasive.

The group working with Dozmorov compared the gene expression profiles of TCC cell lines grown on Matrigel, SISgel and plastic (18). After a microarray analysis of 1186 genes all belonging to the major pathways of cancer malignancies, only 20 genes were identified as being expressed consistently in the cells regardless of the underlying matrices.

The matrix thus greatly influences phenotypic appearance and can even suppress primary cell properties. This must be taken into account, especially in carcinogenesis studies and when oncogenic changes are investigated in cell culture models.

Models and therapy. Therapeutic approaches to TCC include all fields of treatment, *e.g.* chemotherapy, immunomodulation, using BCG or interleukines (19), herbal agents such as hypericin (20) or gene therapy and gene modulators such as antisense oligodeoxynucleotides (AS-ODNs) and small interfering ribonucleic acid (siRNA) (21, 22). To simulate therapeutic procedures, the cell culture is usually incubated with the test agent.

New therapeutic strategies such as the use of AS-ODNs have been evaluated *in vitro* on cell populations. Schaaf and co-workers demonstrated a reduced survival rate in four different human TCC cell lines (UM-UC 3, RT112, T24/83 and HT 1197) after incubation with BCL-2 AS-ODNs and cisplatin as opposed to chemotherapy mono treatment (23). Cell survival rates were measured using a Neubauer haemocytometer or standard MTT assay.

Gazzaniga and co-workers recently published a cell line model for high-risk superficial TCC (24). After treatment with gemcitabine, they used DNA fragmentation, flow cytometry and caspase activation to evaluate appoptosis. Antiapoptotic gene activity was surveyed: BCL-2, BCL-X, BAX, survivin and FAS gene expression were determined by reverse-transcriptase polymerase chain reaction, and nuclear factor-kappa B activation was analysed by immunofluorescence. They found a lack of factor-kappa B activation and FAS up-regulation of gemcitabine treatment compared to other chemotherapeutic agents. Because of these reduced therapy-induced anti-apoptotic activities, they postulated the advantage of gemcitabine in high-risk TCC (24).

Several methods can be used to evaluate results of cell line studies. Common applications are microscopy, immunohistochemistry, immunoassay, Western blot, photometry and FACS analysis. Therapeutic approaches and detection methods both include a wide range of technical options.

Evaluation of cell lines as representative models. The results of cell line studies have to be interpreted as approximations of the clinical situation and thus might include unpredictable discrepancies as regards *in vivo* results.

Cell lines are established from primary tissue by immortalisation or spontaneous proliferation *in vitro*. However, biologically, biochemically and genetically, cell lines might differ from their origins – in their morphology, phenotype or growth characteristics, for instance (25-28). Currently, the information on gene expression and alterations of cell lines in culture is insufficient. High rates of passaging are associated with spontaneous mutations, senescence and selection processes (29, 30). Even though cell lines are established over several decades, the basic cell activities have not been thoroughly investigated and understood. Yet this is essential to know in order to evaluate whether a model is representative and reproducible.

Three-dimensional aggregates reflect *in vivo* conditions more closely (31). They mimic the natural tissue environment while promoting intercellular and cell matrix interactions. The loss of three-dimensional structures in monolayers leads to an alteration of properties and may change the impact of testing agents on the cells. Among other things, this is due to direct exposure, whereas multilayers shield the underlying cell layers (12). Miller and co-workers postulated as much as a 1000-fold decrease in chemosensitivity in one cell line in multilayers compared to monolayers due to tissue architecture only (32). In a recent study, RT112 cells in three-dimensional settings showed a dramatically lower phototoxicity to hypericin compared to cells in two-dimensional settings and the authors suggested that multilayer models be reviewed critically (20).

Tissue culture.

Instead of using abnormal cells from immortalized cell lines, it might be better to use biopsy samples in basic research, as these could well provide models that mimic conditions in humans more closely. For this reason, solid tumour tissue is frequently planted into organ culture systems to form a three-dimensional tumour model. Tumour fragments, taken from a transurethral resection of a bladder tumour (TUR-B) or from cystectomy specimens for example, are immediately placed on an ECM and flooded with medium until the tissue surface is positioned at the interface between the air and the liquid. Only a few different models have been described. Kelly and co-workers placed tumour tissue on a perforated surface that was completely covered with nutrition medium (33). Underneath the surface, they pushed gas through a diffuser so as to continuously produce a foam of medium and gas that fed the tumour samples. The authors reported a significant association between *ex vivo* and *in vivo* tumour response to therapy and results from the model seemed to be able to predict chemosensitivity of the tumours *in vivo*.

There are no set standards for the culture mediums, and so research groups tend to make up their own solutions or present modified models of ones that were published earlier. The lack of standardisation leads to difficulties in comparing the results from different reports. In many models, a commercially sold culture medium is supplemented with serum and a variety of optional supplements such as amino acids, salts, enzymes and drugs. Serum is a vital component in media sustaining cells, with unspecified amounts of assorted conventional hormones and growth factors also being needed at undefined levels. However, FCS added to growth medium has been reported as reducing the viability of TCC cultures. This may well be due to the constituents of foreign serums causing apoptosis in the cultures (34). All culture systems are kept at 37˚C in 5 % CO_2.

Previous studies have shown a success rate of about 70-95% for bladder TCC explants in culture (31, 35, 36). Proliferation, and thus viability, is determined by the cell incorporating labelled nucleosides such as desoxyuridine or desoxythymidine (36, 37). These systems are reported as keeping tissue vital and proliferating for six re-passages and as much as 20 months in collagen gel culture (36). However, Daher and his group found signs of senescence, assessed by the activity of endogenous β-galactosidase and $p16^{INK4a}$ mRNAexpression in tissue after 25 days of culture (37-40). The representativity of the character of tissue cultured for several months is therefore questionable.

Tissue models and therapy. The simulation of therapeutic procedures in *ex vivo* tissue is comparable to the set-up in cell culture models. For tissue incubation, the culture medium is substituted for the test agent, which is often depleted in the culture medium, for a fixed period of time. Many studies focus on the chemosensitivity of the tumours. Burgués and co-workers tested a wide range of chemotherapeutic agents on superficial bladder tumour biopsies (31). Drug sensitivity was determined by using the trypan blue assay. They calculated the response rates for individual agents and were able to assign the best drug to each patient in most cases, enabling *in vitro* drug, dose and time response curves to be plotted. For further investigations of cell activities after treatment, tissue or disaggregated material can be processed for analytical measurements, or preserved in paraffin wax and kryo-conserved. Slices of fixed tissue are processed to enable immunohistochemical staining and microscopic evaluation. The morphology is visible after standard haemalaun-eosin (HE) staining. Apoptosis is a major parameter for anti-tumour activity in the different procedures and can be detected by TUNEL, caspase and Annexin staining.

Evaluation of tissue explants as representative models. In human tissue explants maintained *ex vivo*, *in vitro* and *in vivo* responses will not necessarily be similar because the model has certain limitations, such as the absence of the immunological system and the microenvironment (31). Biopsy samples are unimmortalized cell populations that have a limited proliferative capacity (29). The individual cells within a tissue sample retain the basic metabolic processes, but lose cell functions such as DNA replication and will finally no longer proliferate after a finite number of passages (30). Therefore, tissue kept in a culture process for a long time is questionable with regard to its representative character for *in vitro* systems.

In order to verify the clinical predictive value of results obtained from culture systems, Koshida and co-workers compared the treatment results from tumour samples taken into histoculture with the results obtained from patient treatment (41). Dissected tumour material was grown on a collagen gel matrix exposed to a combination of different anticancer agents. They selected the 20 biopsy samples that showed the most promising results after chemotherapy and applied the same therapy to the corresponding patient. The overall accurate predictive value of the model was reported to be only 55% (42% true-positive results, 75% true-negative results). Thus, results cannot be assigned from *in vitro* to *in vivo* systems without further investigation.

In Vivo Models

Therapeutic animal models.
Animal models are essential in the final preclinical evaluation of new therapies for many human malignancies. Rodents have a similar lower urinary tract to that of humans and neoplasms in the bladder are found to be morphologically very alike (42). Rats and mice are the most common species for *in vivo* TCC models.

The use of chemicals to induce tumour growth in the bladder of rodents takes eight to twelve months, except in the case of transgenic and knockout animals (43). This time-consuming procedure is necessary in order to study carcinogenesis. The animals are exposed regularly to chemical carcinogens, usually given orally and mostly in drinking-water, but injection, intravesical instillation and other methods are also used. Three chemicals have been reported as being particularly effective in causing bladder tumours under the appropriate conditions: FANT (*N*-[4-5-nitro-2-furyl-2-thiazolyl]-formamide), BBN (*N*-butyl-*N*-(4-hydroxybuthyl)nitrosamine) and MNU (*N*-methyl-*N*-nitrosurea) (43).

Tumour implantation is a faster approach to creating therapeutic *in vivo* models for TCC. Within two weeks of implantation, urothelial neoplasms are of a size sufficient for treatment and reproducible evaluation.

Xenograft models, transplantation of human TCC into immunodeficient rodents, are able to form neoplasms that

Table II. *Syngenic rodent bladder cancer models.*

Animal species	Cell line	Tumour cells induced by	Reference
C3H/He mouse	MBT-2	FANFT (*N*-[4-5-nitro-2-furyl-2-thiazolyl]-formamide)	(75)
C57BL/6 mouse	MB49	7,12-dimethylenzanthacene	(76)
Fischer 344 rat	AY-27	FANFT	(77, 78)

are similar in histology and heterogeneity to primary tumours (44).

Syngenic bladder tumours, transplantation of rodent carcinogen-induced bladder cancer in syngeneic, immunocompetent rodents, are needed when research focuses on the immune response, for example in studies on intravesical therapy using immunmodulators such as BCG, and in vaccination, or gene therapy studies (Table II) (45-50).

Tumours in rodents can be induced in the bladder (orthotopically) or subcutaneously (heterotopically). Metastatic growth may occur in several organs. The tumorigenicity of the primary tumour influences the location and successful growth of neoplasms. Prior to any experiment, it is essential to determine which TCC cell lines have caused tumours in what species of animal.

Subcutaneous models. Heterotopic TCC models are located subcutaneously, usually in the flank or hind leg of the rodent. To form a xenograft model, tumour fragments or human TCC cells are inoculated into the immunodeficient host through a small incision. Syngeneic heterotopic models using rodent TCC cells in immunocompetent rodents are described for immunological studies (51).

To date, no stable model has been established for subcutaneous (heterotopic) human TCC xenografts. Primary tumour take rates of up to 100% have been published (52, 53), but success rates for tumour passaging in rodents remain poor (15-38%) (44, 54-56). This passaging is, however, needed for continuing studies, in which individual animals are taken out of a trial and the tumour is transplanted into a new animal.

A recent approach for improving and characterising a xenograft model in severely combined immune-deficient (SCID) mice has been published by Abe and co-workers (57). Twenty-four human bladder tumour biopsies were implanted subcutaneously in SCID mice. A 50% tumour take rate was achieved and it was noted that high-grade tumours grew as solid masses, while low-grade tumours were cystic. Selected genes showed the same mutation status after two passages in xenografts as that analysed in the original tumour, but other genes had a variable range of 50% or higher (57). Another study reported that the higher the grade of the TCC specimen, the higher the tumour implantation rate (54). Grade I samples totally failed to grow in the flank of SCID mice (0/3), but grade IV TCC grew in all animals (2/2). The authors also postulated an association between the topology of TCC xenografts and the primary tumour grade and architecture in the animal. Low-grade (grade II, papillary growth) TCCs were observed as forming fluid-filled vesicles, while high-grade (grade III and IV) samples grew as solid masses.

However, serial passaging of subcutaneous TCC xenografts exhibits selection processes in cell populations as well as morphological changes and a shift in ploidy (44).

Subcutaneous tumours can be evaluated non-invasively by palpation and measuring in addition to image-guided detection. Within one week of tumour cell implantation, neoplasms are detectable. Previous studies have shown the murine MB49 line to be aggressively tumourgenic in mice and that the tumour burden became life-threatening within 3 to 4 weeks after cell implantaion (58). Treatment can be administered systemically, by local injection, *e.g.* intraperitoneally or directly into the tumour, and also by local irradiation (59).

Many studies on subcutaneous bladder TCC are conducted with syngenic models and focus on the immune response. The role of the mutated *RAS* gene as a therapeutic target in MB49 tumours was evaluated in order to develop a specific immunotherapy in case of mutated oncoproteins (58). After tumour implantation, interleukin-12 was administered to the mice in six intervals. The animals were sacrified when the tumours exceeded 2.0 cm. The longer survival time proved that prior immunisation had significantly reduced the growth of the implanted tumours.

Orthotopic models. Orthotopically-grown bladder tumours reflect an environment comparable to the natural appearance of TCC. They are created by tumour cell or tissue implantation into the bladder. Cells are harvested from TCC cell line cultures and a cell suspension is instilled into the bladder after preconditioning or it is injected into the bladder wall (60). Bladder preconditioning, *e.g.* by cauterisation, epithelial abrasion, acidic washing and membrane polarisation, facilitates tumour cell adhesion and improves tumour take rates (45, 46, 61-63). After a period of seven to 14 days, tumour growth should be detectable (64, 65). An overview of different orthotropic models is provided in Table III.

Tumour take was reported to range from 100% (60, 65, 66) to values as low as 28% (46). It is important to track tumour growth continuously in order to evaluate both the initial tumour size and the therapeutically induced changes. Non-invasive quantifications of tumour growth include intravesical ultrasonography (60), *in vivo* magnetic resonance imaging (38) with contrast agents (61, 67), fluorescence-guided coherence tomography (68) and urine

Table III. *Rodent orthotopic TCC models.*

Model	Implantation	Bladder pretreatment	Cell instillation	Growth rate	Detection method	Reference
Orthotopic syngenic bladder TCC in rats	AY-27 cells in CDF344 Fischer rats	0.1 N-HCL, KOH, phosphate buffer solution (PBS)	$1.5x10^6$ cells in 0.5 ml	95% (overall) / 97% (subgroup)	Weekly magnetic resonance imaging	(61) (38)
Orthotopic syngenic bladder TCC in mice	MB-49 cells in C57BL/6 mice	Electrocauterisation	$5x10^4$, $2x10^4$, $1x10^4$, $1x10^5$ cells in 0.05 ml	100%	Gross hematuria, immunohisto-chemically after sacrifice	(45)
Orthotopitc xenograft bladder TCC in mice	KU-7 and UM-UC-2 cells in KSN athymic nude mice	0.2% trypsin in EDTA solution, PBS	$1.0x10^7$ cells in 0.1 ml	100% (KU-7), 80% (UM-UC-2)	Microscopically after sacrification	(65)
Orthotopic xenograft bladder TCC in mice	BIU-87 in athymic nude mice	0.1 N-HCL, 0.1 N-KOH, PBS	$1.5x10^6$ cells in 150 μl	93% (overall) / 95% (subgroup)	MRI, gross pathology, light microscopy	(74)

analysis of markers produced by the tumours of specially designed TCC cells (66, 69). Accurate diagnostics can be performed by means of cystoscopy, providing direct visualization of the intravesical lesions (70).

Invasive bladder investigation is unavoidable in the case of histological staging and grading. The animals are sacrificed and the bladder processed for immunohistochemical and microscopic evaluation. In some studies, fluorescence-labelled TCC cells were instilled into the bladder in order to detect the distribution and quantification across the tissue more precisely (71, 72). A variation of the *in vivo* model is the *ex vivo* model: a group working with Estrada instilled fluorescent TCC cells into the bladder of anesthetised rats for 30 minutes. Afterwards they excised the bladder and urethra en bloc, and the organ was cultured and put into *in vitro* therapy (72).

When an accurate detection method is implemented, therapy simulation can be undertaken and also evaluated. The most frequent procedure used in contemporary clinical practice is intravesical drug instillation. Many therapeutic approaches have been published so far, including photoactive agent application followed by fluorescence radiation to diminish tumour cell growth, oncolytic rheovirus therapy or gene therapy to influence the immune response (47-49, 73).

Conclusion

Many TCC models have been described over the past decades. They vary as to the experimental conditions and basic parameters used, which makes it difficult to compare results. The use of one model for standardised research comes to mind, but is practically not feasible. In the case of cell line experiments, a genetic characterisation might help to estimate whether the results obtained with established cell lines are representative.

There is a definite tendency towards 3-dimensional models for *in vitro* studies. The use of ECM will increase as a result. The advantages that it has over 2-dimensional set-ups are the maintenance of histological structures, cell-cell interactions, and a more physiological environment with regard to chemical and biological gradients.

Apart from 3-dimensional cell cultures, tissue cultures provide different opportunities. Tumours show a great heterogeneity of genotype and phenotype. These qualities are reflected in fresh biopsy samples from a representative patient population. The basic physiological conditions and cellular functions are retained in the fresh tissue. However, samples from a representative patient population are not always available. Reproducibility is hard to achieve because tissues undergo functional changes when being cultured and such changes have not yet been sufficiently investigated.

The medical treatment options for bladder TCC are still insufficient. Better response rates with regard to recurrence and invasion are strongly needed. Protein targeting, vector-based gene transfer or immunization and vaccination have already been proven to reduce tumour burden in therapeutic models and now need to be confirmed in patients. Until the exact genetic mechanism of malignancy in TCC is discovered, therapeutic models will remain the gold standard for investigating new treatment options. Improved characterisation of *ex vivo* and *in vivo* models with regard to molecular alterations in maintained or induced tumours will help to produce comparable and reproducible results.

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