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REVIEW ARTICLE

IN VIVO MODELS IN CANCER RESEARCH

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ABSTRACT

Cancer, which is one of the most dreaded diseases of the century with the number increasing yearly, is a highly complex process which requires various *in vivo* models to elucidate the mechanism of action of the newly developed chemotherapeutic agents and or isolation of lead phytomolecules along with other *in vitro* experiment protocols, before entering into clinical trial phase. This review article deals with some of the *in vivo* cancer models that may help in this process which include various murine models, spontaneous tumour models, laying hen models, drosophila models, chorioallantoic membrane models, zebra fish models etc.

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INTRODUCTION

Cancer is a complex biologic process and a major devastating disease which is influenced by many factors for its development. Cancer studies require comprehensive experimental systems. Cell lines have been used as *in vitro* experimental models to study the biology of cancer cells. Even though the use of tumor cell lines *in vitro* can be highly controlled, unconstrained by ethical considerations, the results are reproducible enabling the validation, repetition and optimization of the experimental assays, yielded many important insights into the molecular mechanisms of cancer, they do not model many features of human cancer because of certain limitations. Most of the cell lines used in cancer research have been generated by serial passages and selection. During this controlled selected growth, cellular transformation occurs frequently with the selection of phenotypic characteristics to adapt to the *in vitro* growth. This selection may include clones with a given set of gene expressions, morphologic characteristics, and functions.

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Moreover, the important role that the tumor microenvironment plays in cancer cell biology is missed in cell line models and therefore precludes the analysis of complex issues within the tumor context. The development in oncology research was made possible by using animal models and these animal models display many complex aspects of human cancer which subsequently lead to the advances in the field of cancer biology and discovery of new therapies.

MOUSE MODELS OF CANCER

Murine cancer models have been extremely useful in the study of the complexity of human cancer, providing valuable insights into cancer biology and biochemistry that cannot be accessed easily by other means. These murine cancer models allow for the three-dimensional growth of tumors with direct interaction with the stromal microenvironment. The generation of mouse models that accurately mimic human cancer must take into account two main criteria: i) the genetic/molecular alterations identified in human cancer and also ii) the target cells where the cancer-mutation takes place in humans. These target cells may be somatic stem/primitive cells, identifying them as the cells to be used as targets in the development of both mouse models and molecular and pharmaceutical therapeutics to treat and prevent human cancers.

This second criterion has not been taken into account in the design of current models of human cancer and may explain why many mouse models of cancer might be inadequate. Numerous agents have shown exciting activity in preclinical models and yet have had minimal activity clinically. These disappointments have led to reasonable skepticism about the true value of both syngeneic and xenograft rodent tumour models in accurately identifying agents that will have important clinical utility. Whereas the development of newer techniques, including transgenic mouse models of cancer, offers the potential to develop more predictive models, the role of such mice in cancer drug development is not yet validated.

Biological criteria to confirm the same genotype phenotype correlations in human and mice need to be used to validate the model, such as:

- Similar histological features to the homologous human tumour.
- Progression through the same stages and equal systemic effects in the host.
- Same genetic pathways should be affected in tumour initiation and progression.
- Response to current cancer treatments should be similar to humans.

Current mouse models

Technical advances over the past two decades now allow investigators to introduce alterations in the mouse genome that constitutively or conditionally alter the expression of crucial genes, leading to the development of particular tumours. These studies have provided tremendous insights into all aspects of cancer research and have further defined the biological functions of hundreds of genes.

Chemically induced carcinogenesis models

Breast carcinoma can be induced in rats by administering dimethylbenzanthracene (DMBA), MNU, and N-ethyl-N-nitrosourea (ENU) and may metastasize to the lungs. Prostate carcinomas can be induced in Noble rats by treating the rats with testosterone/estradiol or MNU/testosterone. An increased incidence of prostate carcinomas can be induced in LobundWistar rats administering methylnitrosourea (MNU) and testosterone. Lung adenocarcinoma can be chemically induced by the administration of vinyl carbamate in A/J mice.

Transplantable animal models

Syngeneic transplantation model

Transplantable tumor models comprise syngeneic models, in which the cancer cell line/tissue transplanted is of the same genetic background as the animal. The advantage of syngeneic models is that the transplanted tissues, the microenvironment (stroma), and the host are from the same species. However, these model systems lack many of the important characteristics of human tumors. For example, they usually are derived from inbred mice and thus lack the genetic complexity of human tumors. Therefore, conclusions drawn from these models should be validated in human cancers.

Xenograft transplantation model

Xenograft models refer to human cancer cell lines/tissues transplanted into immuno-compromised hosts, including BALB/c nu/nu nude and severe combined immuno-deficient (SCID) mice. Although the xenograft models have the disadvantage of an incomplete immune system, a wide range of human samples can be used to study dissemination and colonization, and most mechanistic insight into the process of metastasis is derived from xenograft studies. The evaluation of antitumour agents in immunodeficient mice transplanted with human tumours is the major model system for drug development. In its most simple iteration, tumours are grown subcutaneously, and the model allows rapid and quantifiable assessment of antitumour activity relative to mouse toxicity. Logically, preference should be given to those agents that show the greatest antitumour activity in the preclinical setting, but these preclinical data are not predictive of drug activity in human studies. Xenograft models do not take into account cancer stem cells.

Drawbacks of both transplantable animal models are that only specific stages of the metastatic cascade are represented, as well as the expansion of certain clonal constituents of polyclonal tumors due to cell culture and tissue explanation. Importantly, some crucial features of the tumor microenvironment are lost in these models viz. most of the transplantable tumor models is that the surrounding stroma is 'normal' and not tumor-associated. It has become increasingly clear that primary and metastatic cancers do not exist as isolated tumor cells, but closely interact with different cell types and the extracellular matrix constituting the stroma compartment. Only recently, it has been shown that this heterogeneous and bi-directional interaction within the tumor tissue is responsible for tumor progression.

Transplantation location

Cancer cells can be administered in various ways to small laboratory animals, including inoculation of the tumor cells subcutaneously, orthotopically (at the anatomical site of origin), or at the site of eventual dissemination. Although subcutaneous animal models still remain a valuable approach for tumor progression and metastasis, especially for drug screening purposes, studies on tumor progression and metastasis require a more biologically relevant environment such as the tissue of origin or the tissue to which the tumor cells preferentially metastasize.

- **Orthotopic**

In recent years, considerable effort has been made to develop more clinically relevant models by the use of orthotopictransplantation of tumour material in rodents. It has been shown that it is now possible to transplant tumour material from a variety of tumour types into the appropriate anatomical site and often these tumours will metastasize in a similar manner and to similar locations as the same tumour type will in human cancer. Orthotopictransplantation refers to the delivery of cancer cells to the anatomic location or tissue from which a tumor was originally derived.

The use of orthotopic inoculation has resulted in tumor models that may more closely resemble human cancers including tumor histology, gene expression, responsiveness to chemotherapy and metastatic biology. It is likely that the use of orthotopic systems will strengthen our ability to select the most appropriate molecules for recommended use in clinical studies. Orthotopic transplantation model using murine breast cancer (KEP) cells, murine breast cancer 4T1 cells, PC-3MPro4 cells into the prostrate, Walker 256 rat mammary carcinoma cell lines into the paraspinal area of T12 or T13 via posterior approach.

Current drawbacks of the inoculation of most of the human mammary or prostate carcinoma cells into the murine mammary fat pad or the prostate, respectively, include the lack of an intact immune system and the possibility of tumor cells leaking into the peritoneum following surgery as well as the trauma of opening the mouse peritoneum itself. In order to establish a reliable orthotopic model, sensitive detection of (micro) metastatic spread by molecular imaging is a prerequisite.

- **Subcutaneous**

Inoculation of the cancer cell lines subcutaneously has been another model in cancer research.

Intra- and Supra-Osseous implantation

Other models comprise the inoculation of the cells in the bone, the site to which the tumor cells preferentially metastasize. Intraosseous inoculation results in either osteolytic or osteoblastic lesions or a mixture of those, depending on the cell line used. For example, the breast cancer cell lines MDA-MB-231, MCF-7, and 4T1 as well as the prostate carcinoma lines PC-3, Du-145, and RM-1result in osteolytic lesions. Intraosseous inoculation of human prostate cancer cell lines C4-2B, MDA-PCa-2b, LAPC-9, and LuCaP 23.1 and the breast cancer cell line ZR-75-1 results in osteoblastic lesions.

Another transplantable model of prostate and breast cancer consists of transplantation of human tumor tissue onto the surface of the calvaria. The resulting tumors are moderately differentiated prostate adenocarcinoma with osteolytic and osteoblastic changes that are similar to the histopathological features of human prostate cancer bone metastasis. In addition to prostate cancer, this model has also been applied to study the role of tumor–bone interactions in breast cancer-induced osteolysis and malignant growth in the bone microenvironment. Limitations of these models include the lack of human tumor-to-bone metastasis and the typical location in the bone where metastatic tumors arise. However, this model has proved useful in identifying key factors driving tumor-induced osteoblastic and osteolytic changes such as MMP-7 and MMP-13 (Romero-Camarero *et al*., 2012).

Humanized transplantation model

Commonly used *in vivo* bone metastasis models include syngeneic rodent cancers and xenograft of human cancer in immunodeficient mice.

Species-specific factors from the host (bone/bone marrow stroma) may limit the ability of human cancer cells to metastasize to rodent bones. Important improvements have been made in the generation of preclinical models of human cancer metastasis to human bone. Human fetal bone and adult human rib have been implanted into non-obese diabetic/severe combined immuno-deficient (NOD/SCID) mice, a model called NOD/SCID-hu. Human prostate or breast cancer cells were administered via tail vein injections or directly introduced into the implanted bone. The human cancer cells formed tumors only in the human bone implants and not in the mouse skeleton or in other human or mouse tissues implanted at the same ectopic site.

Hence, these models enable the study of human cancer cell metastasis in a tissue-specific and species-specific manner. Recently, a model was developed based on SCID mice, called the BOM model (human Breast tissue derived Orthotopic and Metastatic model), in which human breast tissue as well as human bone was implanted into the same mouse (Horst and Pluijm, 2012). The human microenvironment of both the breast tissue as well as the bone tissue of this model is important, since species specific differences may determine the interplay between the stroma and the tumor cells. Indeed, it has been shown that the behavior of breast cancer cells in the mouse model was altered in response to variations in the microenvironment.

Dorsal skinfold chamber model

Real-time imaging of single cells *in vivo* can be accomplished by using the dorsal skinfold chamber model. The first transparent dorsal skinfold chambers have been used to monitor angiogenesis *in vivo* with high spatial resolution. In the dorsal skinfold chamber model described by Reeves *et al*. (2010), a metatarsal from a newborn mouse is engrafted into a dorsal skinfold chamber implanted on a SCID mouse. Subsequently, either prostate cancer (PC-3GFP) or breast cancer (MDA-MB-231 GFP) cells are inoculated into the left cardiac ventricle to simulate micrometastatic spread.

The data showed that the osteotropic PC-3 and MDA cells are both capable of homing to the metatarsal within the DSC, whereas oral SSC-4 cells which are known to metastasize to lymph nodes did not. A drawback of these models is the technical skills that are required to the use of the relatively expensive multi-photon microscopy equipment. Because of these issues, it is not feasible to have high numbers of animals included into the experiments.

Systemic inoculation of cancer cells

The experimental metastasis model is a widely used model and refers to systemic inoculation of the tumor cells into the left cardiac ventricle or lateral tail vasculature. The inoculation of cancer cells into the cardiac ventricle is preferred because the number of bone metastasis is higher and the distribution of the bone metastases is superior to that of the tail vasculature inoculation model. Also intracardiac inoculation can be used to monitor cancer cell tropism to specific organs. A potential disadvantage of these systemic inoculation models is that early steps in the metastatic cascade—i.e., carcinogenesis, invasion, and intravasation— are bypassed.

Genetically engineered mouse models (GEM)

While *in vitro* and *in vivo* experimental or 'spontaneous' transplantable models have yielded many important insights into the potential molecular mechanisms of metastasis, a number of important limitations remain. For example, the introduction of cells into the circulatory system bypasses a number of important events thought to be major roadblocks in metastatic dissemination, including escape from the primary tumor, invasion into the surrounding stroma and extravasation. Ectopic or orthotopic implantation, while potentially reintroducing a more natural setting for the process, still suffers from several limitations like the lack of an intact immune system, the inability to model the premalignant neoplastic stages and the surgical procedure itself which may damage surrounding tissue and facilitate the escape of the tumor cells into the bloodstream. This may lead to distant metastasis due to the inoculation procedure instead of tumor growth at the orthotopic site. Moreover, it has been shown that tumorigenesis and metastasis is not just the result of tumor cell characteristics, but rather is a complex interaction between tumor cells and the surrounding stroma. Transplantable models do not necessarily recapitulate all of the interactions between tumor and stroma that may play important roles in tumor dissemination.

Genetic engineered animal models (GEMs), which have a defined genetic background, can be used in immuno-competent hosts and usually have clinically relevant mutations. A number of genetically engineered animal models have been developed.Genetic engineered animal models are valuable because they allow investigators to study the contribution of particular genes to the development of metastasis. They provide flexible manipulation of gene expression at particular time points, thus supporting temporal genetic studies of tumor progression and metastasis. In spite of this, only one or two genes are altered, which is not the situation in human cancer progression. In addition, it is possible that constitutive activation or loss of genes in these models may not completely replicate spontaneous human cancer progression and metastasis. Nevertheless, transgenic mice are important models that are being used to gain insight into the development and treatment of bone metastases. An advantage of these models is the fact that the tumors arise in their normal context and that the animals have a functional immune system. A major limitation of these models is the fact that they are labor intensive and expensive. The current generation of GEMs has a mixed and varied genetic strain background, thus, it is time- and labor-consuming to backcross these lines into a desirable, homogeneous, inbred background before being able to apply them in preclinical trials. In addition, the resources and infrastructure is lacking to consistently generate and evaluate large numbers of GEMs needed for preclinical experiments (Singh *et al*., 2012).

Genetically engineered mouse models have helped to elucidate the molecular pathways involved in oncogenesis, to define the effects of particular mutations or gene deletions on cancer development, and have been useful for validating key genes as targets for therapy. More recently, these models have been used to test targeted therapies, cancer vaccines, preventive agents and combinations of chemopreventive and/or therapeutic agents.

The selective use of GEM models has proved valuable for assessing the *In vivo* inhibitory activities and mechanisms of action of various cancer prevention agents at different stages of cancer development. Genetically engineered mouse models (GEMMs) have proven useful for unraveling tumor cell-intrinsic and cell-extrinsic processes in cancer development and progression, as well as for studying therapeutic responses of autochthonous tumors in an intact microenvironment. The development of tools for spatiotemporally controlled induction of mutations in single cells enabled the creation of GEMMs that accurately mimic sporadic human cancer. Introduction of (combinations of) mutations associated with a specific type of human cancer in the correct cell type in mice often results in tumors that closely mimic the histopathological, molecular, and clinical features of the cognate tumors in patients. Generation of mice harboring human genes involved in processes such as the immune response, drug metabolism, and glycosylation may further humanize mice resulting in models that more closely mimic tumorigenesis and treatment response in humans.

Genetic engineered animal models can be simply classified as either transgenic or endogenous. Mutant mice that express oncogenes or dominant-negative tumor-suppressor genes under control of an ectopic promoter and enhancer elements are called transgenic GEMseg: HBV and HCV transgenic mice models for hepatocellular carcinoma. It involves pronuclear injection into a single cell of the mouse embryo, where it will randomly integrate into the mouse genome.Endogenous GEMs represent mutant mice that either lost the expression of genes or express dominant-negative transgenes or oncogenes from their native promoters. It involves modifying embryonic stem cells with a DNA construct containing DNA sequences homologous to the target gene. Embryonic stem cells that recombine with the genomic DNA are selected for and they are then injected into the mice blastocysts. A drawback of the transgenic models is that it is difficult to obtain the control to express oncogenes at physiological levels. This is important since many overexpressed oncogenes may cause toxic effects including apoptosis and senescence.

Conditional models enable site-specific recombinases such as Cre-LOX and FLPFRT to spatio-temporal control deletion or expression of a gene in specific tissues under control of their endogenous promoter. Models include knock-out miceeg: recently, prostate specific conditional knockouts have been generated of NKX3.1, PTEN, P27, and P53 tumor suppressors that show initiation of prostatic intraepithelial neoplasia and progress into adenocarcinoma with lymph and lung metastasis, in which knock-out alleles replace one ormore exons with a selectable marker resulting in a null allele or knock-in models use transgenes under the control of endogenous promoter and enhancer sequences eg: breast cancer conditionalMMTV-Brca1 model.

The analysis of multiple mutations seen in human tumors is possible by interbreeding GEM to produce mutant mice with both mutations, such as the lobular breast carcinoma model (KEP model) described by Derksen and co-workers. The

results of the simultaneous mutations in the tissue may not reflect the sequential accumulation of mutations in human tumors. This can be addressed by using different site-specific recombinases (e.g., Cre-lox and FLP-FRT) in a temporal manner to produce the relevant mutations. Another aspect is that human tumors are thought to arise from a cell containing one initial mutation, the tumor-initiating cell or cancer stem cell.

The mutations in many GEMs occur in all the cells of the tissue and therefore, the tumor cells do not develop in the context of the 'normal' surrounding stroma. This can be circumvented by the use of Cre-expressing viruses at a low titer, because then the activation or silencing of genes occurs in a few cells, resulting in some mutated cells surrounded by normal cells. This technology can also be used to introduce changes in the stroma.

Sleeping Beauty – genetically engineered insertional mutagenesis system

Sleeping Beauty (SB) is a genetically engineered insertional mutagenesis system. Its ability to rapidly induce cancer in SB-transgenic mice as well as the ease of identification of the mutated genes suggest important roles for SB in the discovery of novel cancer genes as well as the generation of models of human cancers where none currently exist (Howell, 2012). The range of SB-related tumors extends from haematopoietic to solid cancers such as hepatocellular carcinoma.

Molecular imaging modalities

In cancer therapy, *in vivo*imaging has become an increasingly important tool in helping clinicians select patients with the appropriate molecular phenotype for a given therapeutic, provide quantitative information about the optimum biological dose and timing of the therapy (as opposed to the present paradigm of administering the maximum tolerated dose) and assess appropriate biological end points, which may not necessarily be the reduction of tumour size. A variety of small animal imaging technologies have been developed, such as microcomputed tomography (CT), micropositron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), ultrasound imaging and optical imaging, which encompass bioluminescence and fluorescence imaging. Several imaging techniques have already been introduced in a preclinical and—occasionally—clinical setting to assess the presence, real-time growth, invasion, and metastasis of malignant tumor cells.

Spontanous Carcinogenesis Models

Despite the unquestionable importance of these murine models in human cancer research, they are limited in their representation of some essential features that define human cancer, including growth over long periods of time, genomic instability, the function of the immune system, and the significant heterogeneity in tumor cells, tumor microenvironment, and stroma. Another limitation of this model is that sometimes the tumor development and responses observed in mouse models are not predictive of what happens in humans with tumors of the same histology. Furthermore, biologic differences between transplanted cancers in mice and naturally occurring cancers in humans could affect the oncogenesis process. These differences include telomerase that is functionally active in most murine cells, the alterations of certain genes sets and pathways that can vary between murine and human cells, that mice can tolerate higher concentrations of drugs and proteins than human patients, and that their bone marrow may be less sensitive to many cytotoxic agents. In the field of human cancer research, there is a tendency to assess the value of a certain experimental model in terms of similarities with human cancer. Therefore, there is an increasing need for a more appropriate, spontaneous animal model that demonstrates the complex biology of cancer in human patients.

In this regard, companion animals (pet dogs and cats) seem to have many desired characteristics that fill the gap between *in vitro* and *in vivo* studies (Pinho *et al*., 2012). Spontaneous tumors in companion animals, special in canines, are a unique and underused resource as models for human cancer biology and for translational cancer therapeutics. Naturally occurring tumors in dogs have many clinical and biologic similarities to human cancers that are difficult to reproduce in other model systems. The integration of pet animals in clinical trials as preclinical models provides a unique opportunity to evaluate efficacy, pharmacokinetics/dynamics, toxicity, dosing, biomarkers/endpoints, and adverse effects of new drugs before the first in-human studies.

This could contribute significantly to reduce the failure rate of human proof-of-concept studies and, thereby, save time and costs. Many spontaneous tumor types are found in companion animals, such as mammary tumors, osteosarcoma, hemangiosarcoma, lung cancer, skin cancer, prostate cancer, and gastrointestinal cancers among others, have been shown to have an application as human oncologic models.Some strains of rats (LobundWistar and ACI/Seg rats) have an increased incidence of prostate neoplasia. Rodents often develop benign as well as malignant breast cancer. However, most spontaneous breast carcinomas in rodents do not metastasize and have a low incidence of regional lymph node invasion.

The Laying Hen Model

The adult hen is also recognized as a relevant model for human ovarian cancer, because ovarian tumors arise spontaneously in approximately 40% of the hens in later stages of life. The ovarian tumors exhibit serous, endometrioid, mucinous, and clear cell histo-pathological features, express some genes present in human and mouse epithelial ovarian cancer, such as CA125 (*Muc16*), and about 48% harbor mutations in Tumor repressor protein, *Trp53* and an increase in human epidermal growth factor receptor/neuronal tumor gene. Therefore, the hen provides another model in which to determine the progression of this disease and to test various anticancer drugs *In vivo* (Mullany and Richards, 2012).

Drosophila as Cancer Models

Fly approach to exploring cancer mechanisms and even therapeutics is a new *in vivo* model in cancer research. Genetic screens and developmental studies have identified novel Drosophila oncogenes/tumor suppressors and related pathways independent of their known importance to mammalian

tumorigenesis. In many cases, these same genes and pathways were subsequently implicated in human tumors. A small number of solid tumors are dependent on mutations in single loci, including tuberous sclerosis, neurofibromatosis, and Ret-based tumors, such as multiple endocrine neoplasia type 2 (MEN2) and other tumours like human glioma, glioblastoma multiform, colorectal cancer has been studied in Drosophila. Drosophila has also been used to study the tumour invasion and metastasis. Studies in Drosophila have shown that genetic differences between tumour cells and their microenvironment cooperate to promote tumorigenesis (Rudrapatna *et al*., 2012).

Chick Embryo Chorioallantoic Membrane (CAM) Models

Since their introduction almost a century ago, chick embryo model systems involving the technique of chorioallantoic grafting have proved invaluable in the *in vivo* studies of tumor development and angiogenesis and tumor cell dissemination (Ribatti, 2008). The ability of the chick embryo's chorioallantoic membrane (CAM) to efficiently support the growth of inoculated xenogenic tumor cells greatly facilitates analysis of human tumor cell metastasis (Deryugina and Quigley, 2008). During spontaneous metastasis, the highly vascularized CAM sustains rapid tumor formation within several days following cell grafting. The dense capillary network of the CAM also serves as a repository of aggressive tumor cells that escaped from the primary tumor and intravasated into the host vasculature.

This spontaneous metastasis setting provides a unique experimental model to study *in vivo*intravasation step of the metastatic cascade. During experimental metastasis when tumor cells are inoculated intravenously, the CAM capillary system serves as a place for initial arrest and then, for tumor cell extravasation and colonization. The tissue composition and accessibility of the CAM for experimental interventions makes chick embryo CAM systems attractive models to follow the fate and visualize microscopically the behavior of grafted tumor cells in both spontaneous and experimental metastasis settings.The chick embryo chorioallantoic membrane is also commonly used as an experimental *in vivo*assay to study both angiogenesis and antiangiogenesis in response to tissues, cells or soluble factors.

Zebra Fish Model

Zebrafish (*Daniorerio*) represents a powerful model system in cancer research. Recent observations have shown the possibility to exploit zebrafish to investigate tumor angiogenesis (Tohia *et al*., 2011), a pivotal step in cancer progression and target for anti-tumor therapies and a model for normal and malignant haematopoiesis (Jing *et al*., 2011). Experimental models have been established in zebrafish adults, juveniles, and embryos, each one with its own advantages and disadvantages. Novel genetic tools and high resolution *in vivo* imaging techniques are also becoming available in zebrafish. It is anticipated that zebrafish will represent an important tool for chemical discovery and gene targeting in tumor angiogenesis.

Conclusion

The disease being a complex process, the determination of anticancer property based on one step of the several steps becomes difficult. In the history of the animal models for determining cancer therapeutics there are several models that are proved correct and in-correct and efforts are being made to make the perfect one. In spite of the fact that there are no *In vivo* tumour models that completely mimic the human cancer, most of the laboratory studies indicating improved therapeutic responses have been useful to clinical oncologist in improving cancer treatment in man.

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