

Models in Translational Oncology: A Public Resource Database for Preclinical Cancer Research

Claudia Galuschka¹, Romyana Proynova^{1,2}, Benjamin Roth^{1,2},
Hellmut G. Augustin^{3,4,5}, and Karin Müller-Decker²

Abstract

The devastating diseases of human cancer are mimicked in basic and translational cancer research by a steadily increasing number of tumor models, a situation requiring a platform with standardized reports to share model data. Models in Translational Oncology (MiTO) database was developed as a unique Web platform aiming for a comprehensive overview of preclinical models covering genetically engineered organisms, models of transplantation, chemical/physical induction, or spontaneous development, reviewed here. MiTO serves data entry for metastasis profiles and interventions. Moreover, cell lines and animal lines including tool strains can be recorded. Hyperlinks for connection with other databases and file uploads as supplementary information are supported.

Several communication tools are offered to facilitate exchange of information. Notably, intellectual property can be protected prior to publication by inventor-defined accessibility of any given model. Data recall is via a highly configurable keyword search. Genome editing is expected to result in changes of the spectrum of model organisms, a reason to open MiTO for species-independent data. Registered users may deposit own model fact sheets (FS). MiTO experts check them for plausibility. Independently, manually curated FS are provided to principle investigators for revision and publication. Importantly, noneditable versions of reviewed FS can be cited in peer-reviewed journals. *Cancer Res*; 77(10); 2557–63. ©2017 AACR.

Mimicry of Human Cancer

Cancer is extremely heterogeneous with respect to different tumor entities. Overall, cancer researchers are not confronted with a single but with more than 200 different diseases. Furthermore, every individual cancer is unique in its cellular, genetic, and epigenetic composition, and even different areas within the same tumor are heterogeneous. In order to learn how highly mutated and epigenetically modified tumor cells reprogram healthy cells in their environment and at distant sites to serve primary and metastatic growth, *in vitro* and *in vivo* tumor models are used. Some aspects of tumorigenesis can be studied *in silico* (1) or with isolated proteins and nucleic acids (2), genetically homogeneous permanent cell lines (3), and genetically heterogeneous primary cells derived from human patients or animals (4) or organoids (5) growing in culture. Complementary, patient

biopsies are analyzed (6). However, animal experiments turned out to be indispensable to understand gene products driving the hallmarks of the complex evolution of tumor growth (7). The high complexity and diversity translate into a large number of model situations, which recapitulate the human diseases more or less faithfully. Although lower-model organisms, such as yeast (8), *Xenopus laevis* (9), *Caenorhabditis elegans* (10), *Drosophila melanogaster* (11), and zebrafish *Danio rerio* (12), have substantially contributed to the understanding of the molecular basis of the disease, mice are predominantly used. In the future, additional model organisms might come into application due to the technical progress in sequencing and editing the genome.

The broad spectrum of preclinical tumor models ranges from spontaneous or induced tumors in genetically engineered or wild-type organisms, ectopic and orthotopic xenografts or syngrafts, primary human tumor grafts, and tumors induced by full carcinogenesis or multi-step carcinogen approaches, each model type with strengths and limitations. All indications are that a successful translation of candidate drugs into clinical application depends on the treatment success in a battery of models that complement each other to mimic the complex and heterogeneous human tumors histologically as well as molecularly.

Scope, potential use, and benefits of MiTO

Since the number of tumor models is globally steadily increasing, a database with standardized reports to organize, store, and share model data is required. We set out to develop a platform for information exchange about models in basic and translational cancer research, which is called MiTO (Models in Translational Oncology) and is available under <https://mito.dkfz.de>.

The platform aims to deliver an overview about models as comprehensive as possible for cancer researchers including

¹Information Technology, German Cancer Research Center, Heidelberg, Germany. ²Tumor Models, German Cancer Research Center Heidelberg, Germany. ³Vascular Oncology and Metastasis, German Cancer Research Center, Heidelberg, Germany. ⁴Vascular Biology and Tumor Angiogenesis, Medical Faculty Mannheim, Heidelberg University Heidelberg, Germany. ⁵German Cancer Consortium, Heidelberg, Germany

C. Galuschka, R. Proynova, and B. Roth contributed equally as first authors of this article.

H.G. Augustin and K. Müller-Decker contributed equally as senior authors of this article.

Corresponding Author: Karin Müller-Decker, Deutsches Krebsforschungszentrum (DKFZ), INF 280, D-69120 Heidelberg, Germany. Phone: 49-6221-42-4531; Fax: 49-6221-42-4406; E-mail: k.mueller-decker@dkfz.de

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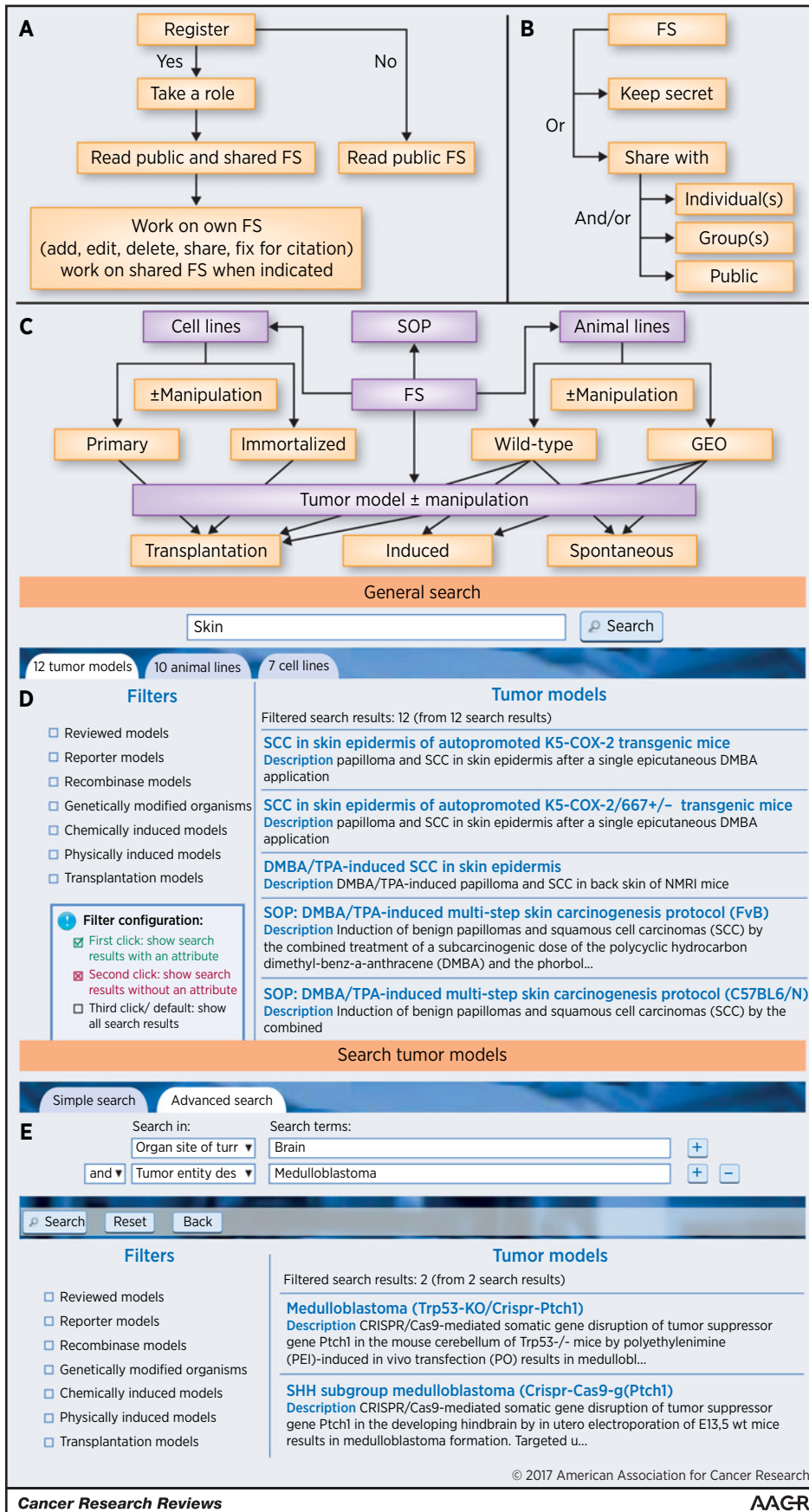


Figure 1.

Key components of MiTO. **A**, Unregistered users can only read FS made public by the owner of a model in the World Wide Web. Registration, concurrent affiliation, and acceptance of a role, e.g., as a researcher, department assistant, or head of department, open the full spectrum of options besides being able to read public FS. Work on own FS involves adding, editing, deleting, sharing, and/or fixing data for citation. When authorized by a principle investigator, work on a shared FS is possible, for example by registered members of a network. **B**, User-defined accessibility of a model FS. Information can be kept secret (private) or shared with other individual(s) and/or group(s) like member(s) of a department, a network, an organization(s). Alternatively, information can be made public in the World Wide Web. **C**, FS are provided for cell lines, animal lines, and tumor models (transplantation models, induced tumor models, or spontaneous models). Metastasis profiles can be implemented into the FS as well as intervention records. Furthermore, SOPs can be documented. **D**, Screenshot of a "General search" for the search term "skin" and the search results separated for tumor models, animal lines, and cell lines applying the filter "only chemically induced models". Here, the list of search results is shown for tumor models, the FS of which can be retrieved via the hyperlinked titles (see also examples of FS in Table 1). **E**, Screenshot of an "Advanced search" within the category of tumor models linking here 2 out of 29 search fields. The search field "Organ site of tumor entity" with the search term "brain" is linked with the Boolean operator "and" to the search field "Tumor entity description/classification" with the search term "medulloblastoma." The list of search results is shown for tumor models, the FS of which can be retrieved via the hyperlinked titles (see also prototypes of FS in Table 1). FS are retrieved as described under **D**.

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Table 1. Key properties of the MiTO database

| Feature | Description |
|--------------------------|---|
| Roles | Department head, department assistant, researcher, administrator, unregistered user. |
| Data availability | User-defined data exchange. The principle investigator (PI) can allow access to a model on the level of reading and editing, separately. Accessibility can be restricted to individuals (to protect intellectual property during model development prior to publication), group(s), one or several department(s) or organization(s), or the public (anybody in the World Wide Web). |
| Data acquisition | Data are generated by registered and affiliated users as well as by MiTO experts by a modified strategy of manual data curation. In the latter case, FS drafts are offered to the PI/owner for revision and subsequent publication under the PI's affiliation. Different descriptions of a model are accepted for better comparison of effects of experimental conditions on the outcome and finding the most robust protocol. |
| Quality check | A model always has to be approved by the author's head of department, first. MiTO experts check for plausibility of entries. In case of inconsistencies, the author is asked for revision. Moreover, each FS is connected to a "report" function, allowing any user to confidentially inform the MiTO experts about putative inconsistencies or problems. Learning tables and drop-down lists minimize misspellings and thereby support search routines. |
| Data search | An unstructured search retrieves all accessible records. The general (simple) search with browsing based on a single query is advisable for a broad finding of all query-related models across all categories (Fig. 1D). The advanced (structured) search with highly specialized queries referring to a distinct field of the FS or combinations thereof allows for a more precise search across a single category. Here, the coupling with the Boolean operators AND, OR, AND NOT, and OR NOT is possible. The application of a single or a multitude of filters serves the positive or negative selection of reviewed models, reporter models, recombinase models, genetically modified organisms, chemically- or physically-induced models, and transplantation models to further specify the results (Fig. 1E). |
| Data content | Search records listed separately for tumor models, animal lines, and cell lines serve the retrieval of FS of individual hits. The tumor model FS considers genetic engineering, transplantation, chemical/physical induction, and spontaneous development and includes a general description, keywords, the animal and cell lines used, tumor types, classification/staging, organs/tissues/cell types of tumor origin, metastasis records, interventions (also in a protocol style to recall SOPs). The FS for animal lines including reporter and recombinase strains contains short name, species, special technologies, genetic background, backcross generation, synonyms, the mutagenesis approach, donor species of the gene(s) and regulatory element(s), modification of gene activity, expression profile in organ(s)/cell type(s), phenotype(s), and additional notes. The FS for primary patient(animal)-derived or permanent cell lines covers short name, special technologies, cell type species, organ, genetic background, synonyms, morphology, disease, and general notes. If an animal and/or a cell line/primary patient-derived cells are used as experimental line(s) in a tumor model, it is linked directly to the tumor model. All FS contain supplementary information as hypertext links to Web-based resources (other databases, references), uploaded files, citable versions, contact data, affiliation of the primary investigator, as well as the dates of first entry and last revision. |
| Fact sheet examples | Cell line: https://mito.dkfz.de/mito/Cellline/11394 Animal line: https://mito.dkfz.de/mito/Animalline/10943 Tumor models: GEO spontaneous: https://mito.dkfz.de/mito/Tumormodel/10553 Transplantation: https://mito.dkfz.de/mito/Tumormodel/10630 Chemically/physically induced: https://mito.dkfz.de/mito/Tumormodel/10474 SOP: https://mito.dkfz.de/mito/Tumormodel/10629 |
| File upload | Per FS 10 files of 4 MB each (larger files or more files on demand) of PDF, JPG, TIF, JPEG, PNG, Powerpoint, DOC, XLS, Sigma plot format. |
| Tools and infrastructure | The web application is hosted at DKFZ on an IIS 8 Microsoft Webserver and was implemented with the Microsoft NET Framework in Visual Studio 2015. Other used frameworks for MiTO are listed here: https://mito.dkfz.de/mito/Home/UsedFrameworks . The database is stored on a Microsoft SQL Server 2008. |
| User support | Access is via the url https://mito.dkfz.de . New features are published directly on the MiTO web site. Support by online tools is via: Web: https://mito.dkfz.de/mito/Help , Email: mito@dkfz-heidelberg.de . |
| Data use | MiTO use is without charge. The commercial use of MiTO or reproduction of any part is illegal without written authorization by DKFZ. |
| Resource citation | Citation of this paper here or by direct reference to the MiTO item: Models in Translational Oncology (MiTO), Deutsches Krebsforschungszentrum Heidelberg, Germany, www.mito.dkfz.de and add month and year of data retrieval. |

newcomers, career changers, successors, etc. A prerequisite for optimal access is registration and affiliation (Fig. 1A). Model accessibility is user-defined and sharing of information/tools can take place at the level of an individual, group(s), department(s), organization(s), or the public, i. e., the World Wide Web (Fig. 1B). To protect intellectual property, information can be kept secret (or private) until publication. However, the major intention of MiTO is to provide information to the public. Serving as a model collector, the tool offers registered users the opportunity to document standard operating procedures (SOP), fact sheets (FS) for cell lines, genetically engineered organisms (GEO), transplantation models including patient-derived xenografts, carcinogen-induced models, spontaneous tumor models, metastasis profiles, and interventions (Fig. 1C). MiTO will facilitate optimal exchange of information and tools, thereby also avoiding unnecessary duplication. Thus, effective exchange is expected to contribute to animal welfare (see the 3R concept) and also to save time and

money. Of note, MiTO FS can be cited with a set of standardized data to complement peer-reviewed articles. Major features of MiTO, i.e., search routines (Fig. 1D and E), roles, availability, acquisition, quality check, and content of data, are described in Table 1 in more detail. To illustrate the structure of the data, hyperlinks to FS examples are available.

Carcinogen-induced models

Conceptually, carcinogenesis is divided into tumor initiation, promotion, and malignant progression. This sequence of events is reflected in experimental multi-step carcinogenesis studies. Carcinogenic polycyclic aromatic hydrocarbons, aromatic amines, N-nitrosamines, aflatoxins and others, as well as physical (e.g., UV-irradiation, ionizing radiation) or viral agents exert potent carcinogenic potential. As compared with full-carcinogenic approaches applying mutagenic carcinogens alone, cocarcinogenesis or multi-step protocols are based on

the combined use of a subcarcinogenic dose of a carcinogen and the repeated application of a nonmutagenic, noncarcinogenic tumor promoter. These agents do not cause cancer on their own, but only in synergism with the activity of a carcinogen.

Standardized multi-step protocols serve to induce murine skin carcinogenesis. Here, mice receive epicutaneously a single dose of the polycyclic aromatic hydrocarbon dimethylbenz-[a]-anthracene for initiation, which produces predominantly via carcinogen-DNA adduct formation irreversible genetic damage along with epigenetic changes. Thereafter, the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate is applied repeatedly, which selectively expands clones of initiated cells by direct activation of protein kinase C signaling, thereby bypassing regulated upstream receptor activation. As a consequence, cells in the tumor microenvironment are also activated and reprogrammed (13). Similarly, protocols using other combinations of stimuli serve to induce cancer in a spectrum of other epithelia such as liver (14) and colon (15). Of note, incidence and multiplicity of carcinogen-induced cancer is mostly strain specific, at least in the mouse.

Such approaches can be applied to animals with different genetic backgrounds and are often used to access chemopreventive measures and to identify risk factors. Although the response of mice and men toward distinct carcinogens is different in many cases and extrapolation from the experimental carcinogenesis study to the human situation needs caution, the relevance of such models has also been documented on molecular level. The characterization of the genomic landscape of carcinogen and genetically induced mouse skin squamous cell carcinoma (SCC) by whole-exome sequencing revealed a significant similarity between recurrently mutated genes in the experimental SCC and human SCC of different origins exposed to different carcinogens (16).

Genetically engineered mouse models

Human cancer in mice is nowadays preferentially modeled by genetically engineered mice. Major reasons for this preference are that 99% and 80% of mouse genes have a homologous or a strict orthologous sequence, respectively, in the human genome and that the technique of experimental embryology, including isolation, *in vitro* culture and manipulation of zygotes, blastocytes, and ES cells and their reimplantation into pseudo-pregnant nurses, are well established in the mouse. The first generation of genetically engineered mouse models (GEMM) produced by classical transgenesis, i.e., microinjection of DNA constructs into the pronucleus of fertilized eggs, is represented by transgenic mice with a tissue-specific compartmentalized gain of function due to overexpression of a randomly integrated oncogene under the control of a tissue-specific promoter, resulting in tumor development in the targeted tissue (17, 18). The second generation of GEM models, known as conventional knock-out by homologous recombination, is characterized by loss of function due to germline deletion of tumor-suppressor gene(s). With this type of mutants, *in vivo* evidence was gained for the tumor-suppressor function of the targeted genes. Unfortunately, such constitutive knock-out models with homozygous germline deletion of distinct tumor-suppressor genes often suffer from embryonic lethality (19, 20) or do not mimic the expected tumor phenotype known from humans (21). To overcome these drawbacks, the third generation of GEM models was developed, which is characterized

by conditional gene targeting. This means, single or multiple genes of interest are inactivated (conditional knock-out) and/or activated (conditional knock-in) upon a temporal- and tissue-specific expression of a bacteriophageal Cre recombinase or a functionally analogous enzyme such as Flp-FRT (Flp recognition target) recombinase that recognize special target sequences for homologous recombination of DNA (22). An even more precise temporal and spatial regulation of gene expression was achieved in modeling human cancer by inducible conditional GEM using hormone-inducible Cre drivers such as CreER (22), regulating gene expression by the tetracycline/doxycycline-inducible system (23) or delivering Cre encoding viruses directly into an accessible somatic tissue rather than the germline (24). These techniques are also applied to introduce reporter alleles for different methods of *in vivo* imaging and lineage tracing *in vivo* (25). The major limitations of these approaches are the long time periods needed to generate and breed genetically engineered compound mutant organisms. To bypass these problems, various strategies evolved to generate multi-allelic GEM models more rapidly, which is also a prerequisite to validate driver genes and potential drug targets in cancer discovered by next-generation sequencing and functional genomic screens. One fast-track strategy is the so-called GEMM-embryonic stem cell (ESC) approach. Here, appropriate GEMMs are selected for ESC derivation. Gene targeting in GEMM-derived ESC is done after the cells have been validated for their potential to contribute to chimeric mice. A recombinase-mediated cassette exchange vector is introduced by a single targeting event in the GEMM-derived ESC, which then allows the further introduction of designer alleles independent of homologous recombination. GEMM-ESC harboring various additional candidate genes are then injected into preimplantation embryos to generate chimeric animals that are directly channeled into experiments (26).

Recently, a completely new strategy for rapid gene targeting was introduced, which is CRISPR/Cas9 (clustered regularly interspaced palindromic repeats/CRISPR-associated gene 9 encoding a nuclease)-mediated genome editing (27, 28), allowing even the one-step generation of mice with mutations in multiple genes (29–31). This technical breakthrough also for the field of cancer modeling allows the introduction of site-specific modifications into the genomes of cells and organisms, irrespective of the species. Derived from a bacterial system of CRISPR/Cas9 that equips bacteria with adaptive defense against foreign DNA, the Cas9 endonuclease tool recognizes the target of interest by a synthetic single-guide RNA. Thereby, the enzyme is enabled to introduce a blunt double-strand break into the target DNA in a site-specific manner. This triggers endogenous cellular repair either along the error-prone non-homologous end joining for loss-of-function mutations, due to insertion or deletion mutations (indels), subsequent frameshift mutations and generation of premature stop codons, or alternatively along homology-directed repair in the presence of an exogenous donor DNA template for gain-of-function mutations and precise gene modifications. Meanwhile, a battery of genetically modified Cas enzymes serves the programmed deletion, insertion replacement, modification, labeling, or transcriptional modulation of DNA of interest opening a new era of genome engineering by this tool (31).

To discriminate cancer driver genes or cancer-relevant noncoding DNA from passengers and to identify oncogenic networks in cancer in a higher-throughput manner on a genome-wide scale, transposon-based approaches have been developed (32). Unique

transposon tools are used for insertional mutagenesis screens in mice. Conditional Sleeping Beauty systems have been developed to drive genomic transposition by a "cut and paste" mechanism in a tissue-specific manner. A promoter-directed and Cre-driven conditional expression of the transposase facilitates random transposon insertion into the genome. Upon insertional activation of a proto-oncogene or inactivation of a tumor-suppressor gene, tumor formation is stimulated. Sequencing data of the most frequent insertion sites allowed the identification of many new driver genes (33, 34). Alternatively, a spectrum of piggyBac transposon insertional mutagenesis-based mouse models for cancer gene discovery have been employed for constitutive, tissue-specific, dominant, or recessive genetic screenings (35, 36).

Cell-based transplantation models

Despite the enormous progress with GEM models, the vast majority of preclinical tumor biology research is still based on rather reductionist cell line transplantation models. These are extensively pursued in basic as well as translational oncology research for validation of cancer genes and for evaluation of the efficacy, pharmacodynamics, pharmacokinetics, and tolerability of candidate drugs. The transplantation of permanent *in vitro* immortalized cancer cell lines, or primary animal/patient-derived tumor cells or tumor pieces into the mouse or rat is done predominantly in an ectopic manner: The cells are inoculated into a tissue that is not representing the organ of origin but a convenient anatomical location or a highly vascularized site, i.e., the subcutaneous space or the subrenal capsule. It is worth to mention that the overwhelming number of ectopic transplantation models do not mimic the human counterpart with respect to tumor vascularization, tumor microenvironment, metastatic spread to the relevant distant sites, or responsiveness toward chemotherapeutics and other drugs (37). Cell transplantation into the organ of origin, the orthotopic transplantation, is rarely done, although it resembles much better the human situation with respect to the tumor microenvironment or cancer metastasis. However, depending on the context, this assay needs advanced technical skills (38).

Human cells/tissues are injected in hetero-(xeno)-transplantation models into a more or less immunocompromised acceptor animal with a different genetic background, mostly mouse. Immunodeficient hosts are a prerequisite in these settings to prevent immune rejection of the foreign cells. Here, the discoveries of the T-cell-deficient athymic Nude (nu/nu) mouse, the T and B cell-deficient SCID, SCID-Beige, and NOD-SCID and Rag1/2^{-/-} mouse strains were a prerequisite to grow human cancer cells. The severely immunocompromised NSG (NOD-SCID gamma) and NOG (NOD/Shi-scid/IL-2Rγ^{null}) mouse strains lacking T cells, B cells, and NK cells and displaying even a reduced activity of monocytes (39) represent recipients of higher rates of human engraftment (40). A major advantage of this approach is that human tumor cells are used to model the human situation, while these models, of course, do not allow assessing the functional contribution of immune cells to tumor formation. If species-specific factors are subject of research, one possible bypass is the use of humanized mouse acceptors. Humanized substrains of NSG and NOG expressing species-specific human cytokines, growth factors, receptors, histocompatibility antigens or adhesion molecules, or harboring even functional human hematopoietic stem cells or tissues such as bone marrow, liver, and thymus are under development to address questions related to human

immune cells and stem cells within their niche (39, 41) as well as virus (EBV and HCV)-related aspects of cancer (42). In contrast, syngeneic, immune-compatible transplanted cells are not rejected by the host organism. The presence of an intact immune system allows investigating the contribution of host immune cells within the circulation but also in the tumor microenvironment to the development of tumors and metastasis. Of note, host organisms are mostly inbred mouse strains and therefore are of less genetic heterogeneity than human patients.

Patient-derived xenograft models

Patient-derived xenografts (PDX), generated directly by inoculation of fresh tumor material, are used nowadays especially in the context of drug discovery and personalized cancer treatment, since they are known to reflect the patient tumor on histologic as well as molecular/mutational level more precise than the cell line-derived xenografts (CDX) that in the majority of cases are based on *in vitro* selected cells. With PDX, the preclinical study results are expected to reflect the clinical response of tumors to a distinct therapy more closely because in a retrospective study, the therapy response in a PDX model proved to have a higher predictive power for therapy response in a distinct patient than the responses from CDX approaches (43). However, the low rate of PDX engraftment and the variable growth kinetics as compared with CDX are still a limitation for their routine use. Another field of application is the characterization of cancer-initiating cell populations by serial transplantation and limited dilution experiments of primary tumor cells.

Cancer metastasis models

Local recurrence and metastatic disease are the primary cause of cancer-related mortality. Models of spontaneous metastasis after orthotopic tumor growth are largely lacking, and better metastasis models are urgently needed not only for basic research but also for the establishment of primary and adjuvant treatment protocols (44, 45). With the steadily increasing number of GEMM, the number of models with spontaneous metastasis in immunocompetent hosts is also increasing, although it may take long for occurrence of metastasis, which is generally asynchronous (46). Also upon ectopic or orthotopic tumor cell inoculation, spontaneous metastasis is a rare event. This is in part due to the simple fact that the primary tumor reaches mostly unacceptable dimensions within a time period that is not sufficient for outgrowth of metastases. Therefore, so-called experimental metastasis assays are performed, in which cells are inoculated via different routes into the recipient organism. For example, tumor cells are injected into the systemic circulation via the tail vein, the left heart ventricle, the intra-portal vein, or the carotid artery, mimicking only the late phases of metastasis, i.e., the survival in the circulation, organ colonization, and local outgrowth. In these colonization approaches, "artefactual" metastasis occurs rather rapidly. Depending on the tumor cell-specific tropism and the site of injection, the "metastatic" nodules grow out predominantly in the lung (tail vein), the liver (intraportal), the brain (intracarotid), or different distant sites including the liver, ovaries, bone, and brain (intracardiac; ref. 47). In an attempt to improve cancer metastasis models, metastasis-competent primary tumors may be resected to allow an outgrowth of spontaneous metastasis, which, as mentioned above for GEMM, also arise asynchronously over a long period of time. The evaluation of the metastatic load is a prerequisite for appropriate randomization prior to drug application.

Here, tumor cells with stable expression of a reporter are advantageous to monitor noninvasively proper resection and more importantly, metastatic disease progression (25). Alternatively, circulating human tumor DNA in the blood of the transplanted recipient mice may be analyzed to determine tumor burden (48).

3Rs: Replacement, refinement, and reduction of animals in research

Achieving scientific results by using experimental animals is as important as animal welfare (49). Especially with respect to GEMM, exchange of existing live mice or cryo-conserved embryos/sperms is highly relevant to avoid unnecessary repetition of the production of a desired mutant. This is particularly evident for recombinase and reporter strains. Consequently, a reduction in animal numbers used to generate a GEMM is expected, and a number of procedures on mice with the potential of distress (superovulation, collection of blastocysts, anesthesia, surgery/vasectomy/embryo transfer, peri/post-operative care, tissue sampling for genotyping, necropsies) are avoided. If a distinct GEMM has already been imported from another institution, a repeated mouse transport along with an eventually necessary embryo transfer procedure to meet the hygiene status of the acceptor facility may be avoided. Giving MiTO users also the opportunity to implement procedures as needed to create transplantation models, protocols for chemical carcinogenesis experiments or inhibitor/diet studies shall facilitate comparison of protocols and help to find appropriate information for desired studies. Hence, MiTO is expected to contribute to the standardization of preclinical tumor models as well as the more efficient use of preclinical models to thereby make an important contribution to the 3R principles of animal experimentation.

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MiTO as a citable standard for tumor models

The development of more standardized data reports of experimental animal studies is urgently needed (50). Therefore, MiTO datasets will continuously be expanded in compliance with international initiatives and associations. Obligatory, FS consist of coherent minimal information of essential nature about a tumor model or a tumor experiment enabling researchers to critically evaluate the data and to reproduce more easily a tumor experiment. The citable FS could turn out to be advantageous, since they can be referenced in publications. Moreover, linking MiTO to relevant biological databases to definitely identify genes, proteins, species, and tumor entities will be enabled. General links to other databases, such as phenotype-genotype or pharmacology structural databases, will also be of primary interest.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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