Primary human tumor xenografted models ('tumorgrafts') for good management of patients with cancer

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The treatment of cancer is continually improving as a result of a better understanding of oncogenesis and the development of new targeted compounds. Early clinical trials evaluating such candidate compounds require a large number of patients, and are expensive, time consuming, and expose patients to certain risks. To select the most effective molecules, preclinical investigation of antitumor compounds is an important step in the drug development process. Three main categories of preclinical cancer models are generally used in preclinical investigations, namely genetically engineered models, xenografts derived from human tumor cell lines, and human tumor fragments from patients implanted directly into immunodeficient mice, known as tumorgrafts. The establishment of tumorgrafts constitutes a long-term process consisting of various steps, in which the final objective is to show that the validated model accurately reproduces human cancer, with a high predictive value of therapeutic efficacy (regardless of the type of treatment), and closely mimics clinical situations frequently observed in patients with cancer, such as resistance to standard treatments, metastases, and relapse after initial therapies (involving residual tumor-initiating cells). The aim of this study is therefore to discuss the proposed criteria for the establishment and validation of preclinical models of human cancers that should be used for further pharmacological assessments. ***Anti-Cancer Drugs*** **22:827–841 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The continuously growing body of knowledge with regard to oncogenesis, allowing identification of new molecular pathways and therefore new potential targeted compounds, increases the efficacy of antitumor treatments and improves the outcome of patients with cancer. However, the very large number of therapeutic candidates would require a large number of clinical trials to assess their efficacy compared with standard treatments and/or in combination with standard treatments, to define their optimal schedule(s) of administration and toxicities, and to identify predictive markers for response and/or resistance. Owing to unexpected and unacceptable toxicities that may occur in patients during early clinical trials, which are also time consuming and expensive, it is of importance that new compounds are strictly selected. To discriminate the potentially most effective and most specific molecules, preclinical investigation of new compounds therefore constitutes an important step in the drug development process, underlying the essential role of relevant tumor models. The choice of preclinical tumor models used to evaluate new compounds is a critical determinant to obtain preclinical results with a high predictive value for further clinical trials, because, in addition to the molecular pathways specifically targeted by the new candidate compounds, characterization of tumor cells, including histopathological, molecular, genomic, transcriptomic, and other analyses of tumor vascularization and stromal components, as well as the intratumor distribution of the new pharmacological compounds, strongly determine their therapeutic efficacy, and therefore their future clinical usefulness and indications.

Three main categories of preclinical cancer models are generally used in preclinical investigations, namely genetically engineered models (GEMs), xenografts derived from human tumor cell lines, and human tumor fragments obtained from patients, directly transplanted into immunodeficient mice, without an intermediate in-vitro passage, and recently called 'tumorgrafts' [1]. More confidentially, humanized models that allow the growth of human tumor cells in a human environment in mice have been reported in breast cancer (BC) models [2,3]. GEMs and xenografts, and particularly tumorgrafts, are defined by specific histopathological and molecular characteristics that allow them to be used for evaluation of new targeted compounds [4]. Each of these models also has theoretical and practical advantages and disadvantages that could be summarized as follows: tumorgrafts, derived from human tumors, accurately reproduce the marked heterogeneity of human cancers, particularly when large panels of a specific tumor type are available. Furthermore, procedures for assessment of therapeutic efficacy have been well standardized and readily allow evaluation of combined therapies, particularly

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for the purposes of objective biostatistical assessment. Finally, the possibility of ex-vivo genetic or therapeutic manipulations before xenotransplantation constitutes an argument in favor of the use of xenografted models. In contrast, orthotopic transplantation is rarely possible and interscapular xenografts are therefore usually performed, raising doubts about the development of tumor blood supply and tumor–stroma interactions similar to those observed in patients with cancer. GEMs often concern specific molecular targets highly suited to preclinical assessment of the corresponding targeted compounds, as tumor growth occurs spontaneously *in situ* and in organ-specific sites. This reproduces the situation observed in human cancers and interactions between tumor and stroma, particularly during neoangiogenesis, as well as the impact of the immune system, appear to closely mimic human situations. However, assessment of therapeutic efficacy could be hampered in the case of nonsuperficial or delayed tumor growth and by the fact that the GEMs represent only one model of the tumor-specific target, which does not necessarily predict that the target itself will be coupled to the biological 'read-out' in real human cancers [5]. Moreover, the variable penetrance of the modified gene argues against their use for 'screening' purposes.

To evaluate the relevance of tumorgrafts as useful tools for preclinical studies, one approach consists of defining the various clinical and oncological issues that must be resolved as completely as possible by these models. Human preclinical tumor models must therefore (i) accurately reproduce human cancers, individually between the tumorgraft and the original patient's tumor, and collectively between the panel of tumorgrafts and the various subcategories of human cancers, (ii) have a high predictive value of the therapeutic efficacy (regardless of the type of treatment) observed in patients with cancer, with a high correlation between preclinical and clinical results, and (iii) closely mimic clinical situations frequently observed in patients with cancer, such as response and resistance to standard treatments, metastases, and relapses after initial therapy, raising the issue of the possible role of residual tumor cells after treatments and/or tumor-initiating cells. The aim of this study is not to compare the main categories of preclinical cancer models or to present methodological issues of preclinical experiments, which are well detailed in a recent study by Hollingshead [6]. Instead, the study aims to discuss, in the setting of primary human preclinical xenografted tumors, the various criteria that could be defined for the establishment and validation of preclinical models to be used for further pharmacological assessments.

Reproducibility of the models

Reproducibility of the tumorgraft compared with the original patient's tumor

At the time of molecular diagnosis of human cancer that currently may impact the patient's subsequent treatment, characterization of the tumorgraft constitutes the first essential step of validation of the model. It is essential to determine all tumor characteristics involved in the management of patients with cancer, continuously updated by new published biological data. Preclinical trials should be primarily based on standard clinical practice. A striking example is screening for *K-ras* gene mutation before epidermal growth factor receptor (EGFR) targeting [7]. Validation of the model therefore requires various types of assessment according to the type of cancer (i.e. BC, colon cancer, glioblastoma, etc.) and according to subcategories of cancers (i.e. BCs, basal-like BC, normal breast-like BC, luminal BC, and ERBB2 + BC) [8]. The minimum initial characterization of tumorgrafts should also include histopathological and molecular evaluations, and as far as possible must be completed by genomic and gene-expression profile studies. All these characterizations should be performed concomitantly for the tumorgraft and for the corresponding patient's tumor. A pathologist experienced in human cancers is required to perform these specialized examinations on both the tumorgraft and the patient's tumor.

The pathologist must evaluate whether or not the xenograft reproduces the features of the original tumor, by examining the appearance of the tumor cells and the pattern of proliferation. It should be noted that a great majority of tumorgraft models have been developed in nonorthotopic situations, which therefore cannot reproduce spontaneous tumor cell growth in an in-situ organ-specific site. Patient tumor samples are almost always obtained at clinical stages eligible for surgery, allowing ethical and research-motivated use of malignant tissues considered by the pathologist to be organic wastes. In contrast with GEMs that may confirm the role of specific genes during the oncogenetic processes, for instance breast cancer gene 1 (*BRCA1*) for BCs [9,10] and adenomatous polyposis coli gene for colon cancers [11–13], tumorgrafts are not useful tools to determine the mechanisms by which a normal cell is transformed into a malignant cell. It has been commonly observed that, despite the nonhuman stromal cells and blood supply, the histology of the original tumor is generally conserved in tumorgrafts, as the morphology of cancer cells, the abundance of stroma, and necrotic areas are similar in both tumors [5,14] for a wide variety of cancers such as BC [15–17], uveal melanoma [18], ovarian cancer and uterine sarcoma [19,20], soft tissue sarcoma [21], pancreatic adenocarcinoma [22,23], colon cancer [24], non-small cell lung cancer [25–27], small cell lung cancer [28], brain tumors [29], and acute lymphoblastic leukemia (ALL) [30,31].

In contrast, several reports of soft tissue sarcomas have reported phenotypic variability [32,33], particularly on the basis of global analysis of all primary tumors and their corresponding xenografts with a large panel of immunomarkers [33]. In particular, several differences are conspicuous when comparing the ultrastructure and

differentiation of tumorgrafts and original tumors [21]. Indeed, soft tissue cancer cells may differentiate in various directions of mesenchymal differentiation after appropriate stimuli or after selection of less well-differentiated cells during the transplantation process [34]. Similarly, we have observed several cases of apparent discordance in terms of cell components in uveal melanoma xenografts, that is, epithelioid, spindle, or both cells, suggesting that in-vivo tumor growth of heterogeneous tumors may possibly concern only one tumor cell component [18]. Although no published study has specifically evaluated the development of tumorgraft vascularization, many papers have assessed tumor vasculature using histopathological protocols (number of blood vessels per unit area by immunostaining endothelial cells for von Willebrand factor, CD31 or CD34) [35] and/or imaging studies (microscopic imaging methods, optical imaging techniques, and clinical methods such as magnetic resonance imaging and positron-emission tomography). The study that evaluated vascularization of xenografted pancreatic mouse tumors and GEMs should be emphasized [36]. This publication highlighted the role of the peritumor stroma that might influence therapeutic efficacy and the preclinical relevance of the model. It is therefore evident that both vascularization and stromal characterizations of tumors would increase the accuracy of preclinical therapeutic assessment of new therapeutic approaches.

Molecular analyses constitute a crucial step in the characterization of the model. These analyses should be defined according to the type of cancer and must allow easy comparisons between the tumorgraft and the original patient's tumor to confirm the similarities between the two tumors. All molecular markers obviously cannot be defined in the context of this study. However, two main criteria can be proposed to define the list of molecular markers that should be evaluated. First, molecular markers may have a potential role in oncogenesis, that is, early and late events leading to cancer initiation, progression, and dissemination. Second, molecular markers may have a potential impact on both conventional and innovative therapies in terms of clinical therapeutic indications and response/resistance to treatments. In both situations, molecular markers may change continuously with progress in our knowledge: determination of some molecular markers is essential, whereas others may be strongly recommended, or only optional, in which case, they are defined according to local priorities, projects, and opportunities. Such an order of priority should therefore be established cautiously at the beginning of tumor characterization and updated as necessary. To create useful preclinical models, molecular markers must be defined for each type of cancer in close collaboration between clinicians, pathologists, and research scientists. Moreover, when patient tumor samples are available, new updated molecular markers should be evaluated in both tumorgrafts and the corresponding patient's tumors. For instance, the essential molecular markers in BCs are proliferative index (Ki67), estrogen α and progesterone receptors, and Her2 expression, to classify all studied samples into the four major BC molecular subtypes (ER+ HER2−, ER+ HER2+, ER− HER2+, and ER− HER2) that constitute the basis for determining standard treatment options in newly diagnosed patients with BC [37]. Strongly recommended markers are EGFR expression, p53 mutation, PI3K pathway that plays a crucial role in BC tumorigenesis by PTEN, Akt, p-Akt, and/or mTOR expression, and PI3K mutation. In contrast, the list of optional molecular markers is obviously long and therefore cannot be presented in detail in this study. Gene-expression characterization including the 306 genes allowing discrimination of tumor samples between the five previously identified BC molecular subtypes (luminal A and B, basal, Her2, normal) might be of interest to more accurately classify BC xenografts [38]. Finally, in the context of regular updating of molecular markers in panels of xenografted tumors, tissue microarrays for immunohistochemical analyses could be established, ideally with both preclinical models and their corresponding patient's tumors.

Cytogenetic and genomic analyses (comparative genomic hybridization microarrays or single nucleotide polymorphism arrays) of both the tumorgraft and the corresponding patient's tumor constitute a necessary step of initial characterization to address two distinct fundamental issues. First, are the cytogenetic/genomic abnormalities present in the human tumor also found in the preclinical model? Second, are the genomic profiles similar between the tumorgraft and the original patient's tumor? The first issue is of particular importance in the presence of alterations that possess an oncogenic role and/or prognostic value. For instance, chromosome 3 status, which has a major prognostic impact on the outcome of patients with uveal melanoma [39], is one of the various abnormalities that may be evaluated [40]. In our experience, on the basis of 14 cases for which both xenograft and patient tumors were studied, 10 tumors presented loss of heterozygosity of chromosome 3 (either monosomy or isodisomy) and four had normal chromosome 3 status [18]. A complete concordance was observed between patient tumors and their corresponding xenografts. The four tumors with disomic and heterozygous chromosome 3 led to xenografts with a similar chromosome 3 status. All chromosome 3 monosomic tumors led to monosomic xenografts. Interestingly, the two chromosome 3 isodisomic tumors led to monosomic xenografts. Similarly, focusing on two specific markers (*K-ras* gene mutation and Dpc4 expression) in 12 models of pancreatic carcinoma, Rubio-Viqueira *et al.* [23] showed a high degree of concordance between primary originator and xenografted tumors. Finally, a high level of stability has been observed in various cancer types including BRCA1-mutated ovarian

carcinoma [20], glioblastoma [41,42], BCs [15–17,43,44], synovial sarcoma [33], and ALL models [30]. In contrast, it has also been shown that tumor grafts may develop additional abnormalities to those observed in the patient's tumor [41]. It is noteworthy that genomic stability has been observed in tumor grafts during serial transplantation despite the presence of BRCA1/2 mutations [43,45,46]. The second issue raises two very important questions, namely (i) are genomic profiles modified during the in-vivo transplantation process? and (ii) are genomic profiles modified during in-vivo maintenance of the model? Despite the extensive use of primary human xenografted tumors, very few published studies have evaluated genetic/genomic stability between tumorgrafts and the original patient's tumor and between various serial passages of in-vivo transplantation. As growth rates may increase during the first few transplant generations, they are maintained within the same host [47]. Comparison of genomic profiles between tumorgrafts and original tumors in four types of cancer, that is, BC [44], gynecologic tumors [46], pancreatic adenocarcinomas [48], and glioblastomas [41], showed that both samples matched in the same cluster, suggesting genetic stability during the in-vivo transplantation process. Finally, gene-expression profiles have been compared in human and murine BC samples [17,44], which shows a high level of conservation of tumor gene expression and downexpression of genes corresponding to immune response, response to wounding, extracellular matrix component, cell adhesion, and angiogenesis, all expressed in stromal cells that are human in the original tumors and murine in tumorgrafts. This observation emphasizes the interaction between human tumor cells and the host. It might be interesting to compare the gene-expression profile of stromal cell between tumorgrafts and the corresponding patient's tumors. Finally, an original report showed that, as the immunophenotype of xenografts closely resembled their original tumor, expression profiling of human microRNAs clustered separately from the corresponding patient tumors, but these data need to be confirmed in other series [26].

Reproducibility of tumorgraft panels in relation to human cancer

With the continuously growing knowledge of human cancers and their various subtypes, the initial homogeneity has been completely lost and each organ-specific tumor is increasingly divided into highly heterogeneous subcategories. Molecular markers, genetic abnormalities, and gene-expression profiles are currently used to define new subsets of cancers that require specific diagnostic and therapeutic management. In many situations, such a classification is mostly induced by the origin of cancer cells, particularly according to the architectural and histological organization of the organ from which the tumor arises. Particular examples of this classification process are of malignant lymphomas and BCs, for which the identification and classification have evolved for more than 40 years. Although the treatment of cancers is now impacted by molecular characterizations, efficient local therapies such as surgical excision and/or radiotherapy are and will be included for a long time in the global therapeutic strategies of patients with cancer. Modern preclinical pharmacology should therefore focus on both specific targeted treatments and organ-specific cancer cells. Moreover, in some situations, the various subcategories of cancers are defined by molecular markers, as in the case of BCs, which can have an impact on treatment. Finally, the efficacy of new targeted therapies may be influenced by molecular markers, and these new compounds therefore need to be evaluated on a wide range of tumor models that accurately reproduce all subcategories of human cancers. The search for molecular markers predictive of response and resistance requires objective and unbiased screening for which a panel of tumorgrafts representing various subgroups of cancers would therefore be particularly useful.

However, the creation of a panel of tumorgrafts that includes the various well-defined subcategories of human cancers is not as simple as it seems for two main reasons: first, the 'tumor take' is highly different with regard to histopathological subgroups of cancers; and, second, tumors that are able to grow in mice frequently originate from patients who are of poor prognosis for both relapse-free and overall survival. Considering the first reason, in our experience, 'tumor take' is high for colon and lung cancers ($>50\%$), intermediate for ovarian cancers and uveal melanoma (35%), low for BCs (9%), and particularly weak in prostate cancers (PCs) ($<1\%$). Considering the second reason, ALL blast cells have a higher in-vivo tumor growth rate in the case of relapsed or refractory disease than in newly diagnosed disease [31]. We have also made such an observation in uveal melanoma tumorgrafts [18]. Similarly, the BC tumorgrafts initially obtained in our laboratory are mainly 'basal-like' (83%) [15], but this proportion is very different from that reported in patients with BC [8]. A panel of tumorgrafts would be very useful to evaluate new treatments in 'basal-like' BC, but such a panel appears to be inappropriate to define markers of response and resistance between the various subcategories of BC. However, despite a very low growth rate of patient tumor samples in mice, namely 9% [15], BC management offers an optimal situation to obtain a more representative panel of tumorgrafts. As shown in Fig. 1, the initial diagnostic tumor biopsy performed in patients with BC allows identification of those cases in which tumor samples are warranted, namely Her2-positive BC and luminal estrogen receptor-positive BC. By using such a strategy, we are currently modifying the proportions of the various subcategories of BC that finally grow in mice (unpublished data), and more than 500 patient tumor samples have already been transplanted at the Institut Curie. This procedure, which

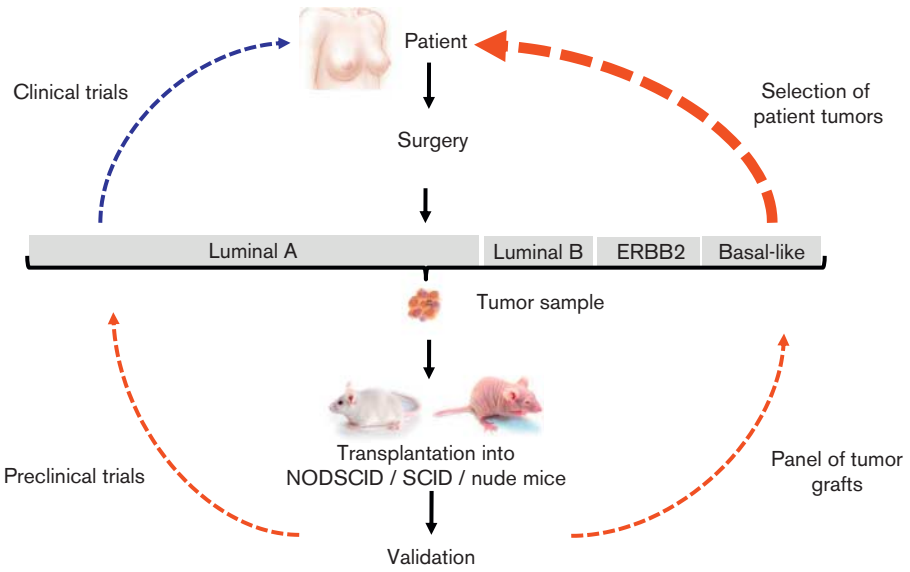
is efficient but constraining, highlights the need for close collaboration between surgeons, pathologists, and preclinical research scientists.

Predictive ability of the model

The second main issue that must be addressed by human preclinical tumor models is to ensure a high predictive value of clinical efficacy of standard and innovative treatments. Two approaches can be used: the first compares the therapeutic response of the tumorgraft with that of the corresponding patient's tumor, and the second compares the therapeutic response of a panel of tumorgrafts with that of a cohort of corresponding patients with cancer.

The first approach has rarely been reported in the literature, probably because of the difficulty of comparing these two different situations. However, several studies have tried to perform this type of evaluation, as shown in Table 1. In brief, a close correlation between tumor response in the patient's tumor and the corresponding xenograft in nude mice has been observed in BCs [15], colon cancers [24], sarcomas [51], ALL [31], ovarian cancers [49], and various other tumors [50,52]. Treatments could be the same or nearly the same in both clinical and preclinical circumstances, namely chemotherapy, radiotherapy, monoclonal antibody, and other targeted compounds, but preclinical and clinical situations are characterized by three main differences, namely (i) patients are frequently treated by a combination of treatments that precludes evaluation of one specific therapy, (ii) patients may present various tumor sizes and sites in contrast with the homogeneous preclinical experimental model, and (iii) the response criteria are very different between patients and transplanted mice. In the clinical situation, the endpoint is dependent on whether patients with cancer are treated in an adjuvant setting, that is, in complete remission (CR) after initial surgery, such as in patients with BC, or in a neoadjuvant situation with an evaluable tumor mass. In the first case, the endpoint will then be progression-free, metastasis-free, relapse-free, event-free, disease-free, and/or overall

Fig. 1



Development and adjustment of a panel of tumorgrafts. The initial diagnostic tumor biopsy performed in patients with breast cancer allows the identification of cases in which tumor samples are justified, such as Her2-positive breast cancers and luminal estrogen receptor-positive breast cancers, and adjustment of the panel of tumorgrafts finally obtained. NOD, nonobese diabetic; SCID, severe combined immunodeficient.

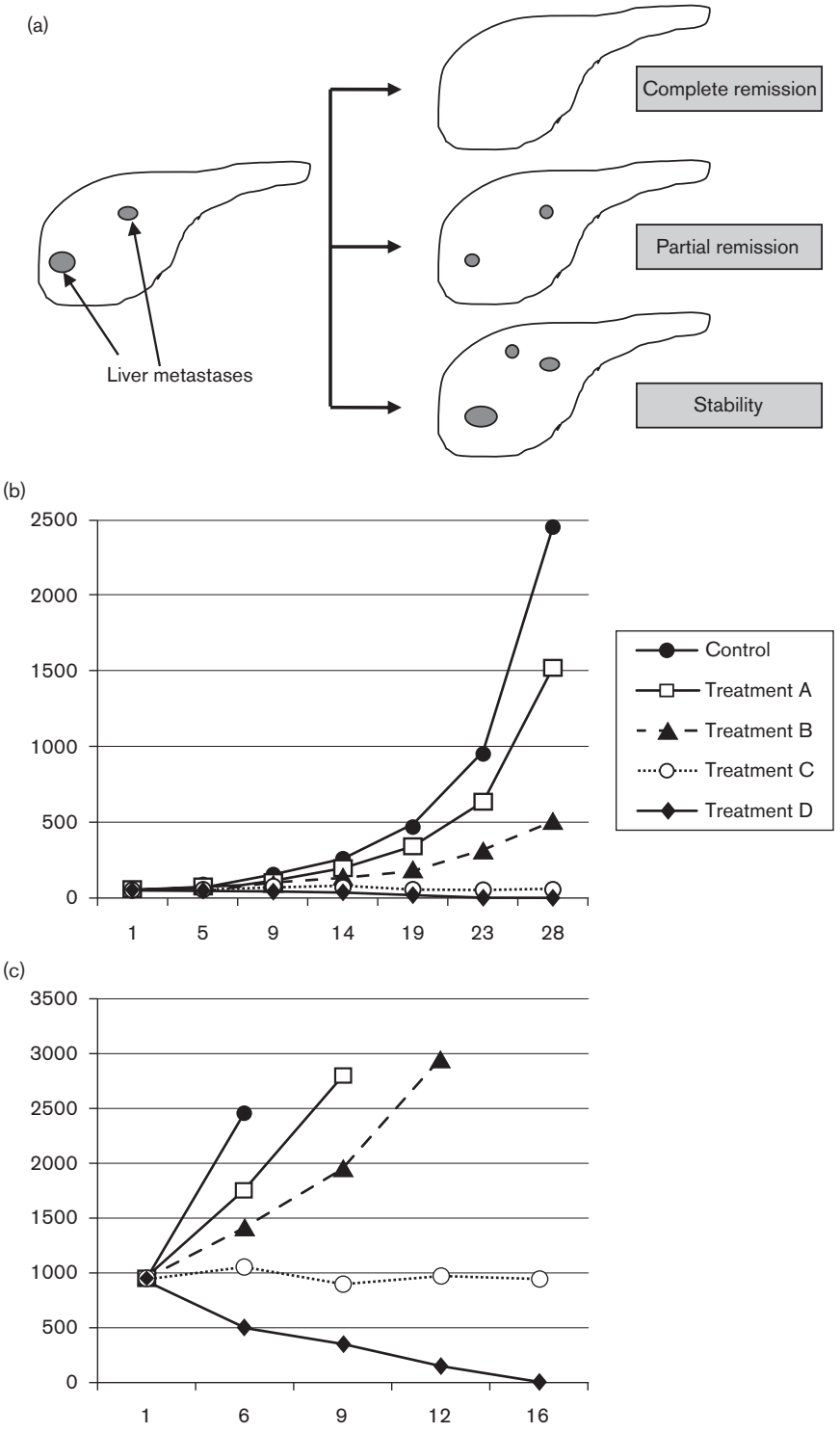
Table 1 Correlations between clinical and preclinical responses in patients and corresponding tumorgrafts

References	Types of cancer	Treatments	*N*	Concordance (%)	Comments
Fiebig *et al.* [49]	Various	Various	50	96	[a]
Sakamoto *et al.* [50]	Various	Mitomycin, doxorubicin, cisplatin	11	73	[b]
Hoffann *et al.* [48]	Sarcomas	Doxorubicin, mitoxantrone, vincristine; cisplatin, ifosfamide, bleomycin	9	67	NA
Harrap *et al.* [51]	Ovarian cancers	Platinum-based chemotherapy	9	89	NA
Marangoni *et al.* [15]	Breast cancers	Doxorubicin + cyclophosphamide	7	71	NA

N, number of preclinical/clinical comparisons; NA, data not available.
[a]Ninety-two percent prediction of response and 97% prediction of resistance.
[b]Overall accuracy of prediction = 95%.

survival, whereas in the second case, it will be the direct response to therapy. The read-out of in-vivo experiments, which will be inhibition of tumor growth, growth delay, and sometimes overall survival, would therefore constitute the best marker of clinical efficacy of treatment administered in a neoadjuvant setting. Similarly, patients

Fig. 2



with metastatic cancer appear to be fairly similar to tumor-bearing mice. However, as recently discussed by Singh *et al.* [52], another discrepancy is observed between clinical and preclinical response criteria: in clinical trials, on the basis of the use of Response Evaluation Criteria in Solid Tumors criteria [53,54], partial response is defined by a decrease of at least 30% in the sum of the longest diameter of all target lesions, whereas in preclinical trials, a partial response is defined by tumor growth inhibition of at least 50% of the control group (Fig. 2). Consequently, preclinical response would often be considered to be progressive disease in clinical trials, and inversely, clinical response would always be defined as a highly responding xenografted tumor. To align clinical and preclinical response criteria, it would probably be better to treat xenografted mice with the largest tumors, but this approach would be associated with a risk of selecting more rapidly proliferative and more resistant tumors that would rapidly reach the tumor size requiring ethical killing of the animals. Moreover, Singh *et al.* [52] suggested that overall survival could be the best endpoint for evaluation of treatment, but it may not accurately reflect tumor growth under treatment and show occurrence of complete responses and recurrences. Overall, this discrepancy therefore suggests that comparison of response to treatment between a tumorgraft and the corresponding patient's tumor is not completely relevant and useful, and that new methodological approaches are required.

The second approach, comparing the sensitivity of human primary tumors in mice and the sensitivity of the same type of tumors in patients, has been largely reported in the literature, using various conventional and more innovative chemotherapeutic agents, and in a large variety of human cancers, including BCs [15,55–61], ovarian cancers [19,51,58–62], colon cancers [55,58, 59,61], non-small-cell lung cancers [25,27,58–61,63], small-cell lung cancers [28,58,59], pancreatic carcinomas [64], melanomas [51,55,58–60,65], head and neck cancers [58,59], brain tumors [60,66], kidney tumors [60], gastric cancers [60,67], and neuroblastomas [68]. Various response criteria have been used for both preclinical and clinical assessments, and different cutoffs of response have been used in models to more accurately predict clinical efficacy. For instance, the National Cancer Institute found that compounds that were active in at least one-third of all human xenografts tested were likely to have at least some activity in phase II clinical trials [60]. Such variability in the definition of response criteria therefore constitutes one of the major criticisms of all of these comparisons. Moreover, comparison of clinical and preclinical phase II studies is hampered by the fact that many patients who entered into clinical trials have been previously treated by other agents and have relapsed with refractory disease, and these patients are more resistant to new therapeutic approaches [69]. However, on the basis of all reported data, the preclinical response of tumorgrafts to conventional chemotherapeutic agents appears to be closely correlated with retrospective or prospective clinical results. In our experience, we have confirmed this observation in two particular examples: (i) 'basal-like' BCs, for which we have observed significant tumor growth inhibition with a combination of doxorubicin–cyclophosphamide in 11 of the first 14 validated models (79%) [15]. This response rate was closely correlated with clinical results showing an overall response rate of 85% after similar combination chemotherapy [70]; and (ii) small cell lung cancer tumorgrafts with a significant tumor growth inhibition of 75% in eight preclinical models after a combination of etoposide and ifosfamide [63] and an overall response rate of 76% in a recently reported prospective clinical trial using the same treatment regimen. Moreover, in a series of 80 comparisons between clinical and preclinical tumor responses, Fiebig *et al.* [71] observed correct prediction of tumor sensitivity in 90% (19 of 21 tumors) and correct prediction of tumor resistance in 97% (57 of 59 tumors) [72]. A direct consequence of this close preclinical/clinical correlation is that clinical trials can be conducted by using

Clinical and preclinical evaluation of response criteria. For clinical therapeutic trials, (a) Response Evaluation Criteria in Solid Tumors evaluation of liver metastases was performed. Complete remission, partial remission, and stability of liver metastases are shown. (b and c) For preclinical therapeutic trials, mice bearing growing tumors were individually identified and randomly assigned to the control or treatment groups (eight to 10 animals in each group) and treatment was started on day 1. Tumour-bearing mice were killed when the tumor volume reached 2500 mm^3, defined as the ethical limit. Tumor volumes were calculated by measuring two perpendicular diameters using a calliper. Each tumor volume (V) was calculated according to the following formula: $V = a \times b^2/2$, where a and b are the largest and smallest perpendicular tumor diameters. Relative tumor volumes were calculated from the formula: relative tumor volume (RTV) $= (V_x/V_1)$, where V_x is the tumor volume on day x and V_1 is the tumor volume on initiation of therapy (day 1). Growth curves were obtained by plotting mean RTV on the y-axis against time (x-axis, expressed as days after initiation of treatment). Antitumor activity was evaluated according to tumor growth inhibition and calculated according to the following formula: percent of growth inhibition $= 100 - (\mathrm{RTV}t/\mathrm{RTV}c \times 100)$, where RTV$t$ is the mean RTV of treated mice and RTVc is the mean RTV of controls at a given time when the antitumor effect was optimal. Fifty percent tumor growth inhibition was considered to be the limit of a meaningful biological effect. The statistical significance of differences observed between individual RTVs corresponding to the group of treated mice and the control groups was calculated by paired Student's t-test. In (b), xenografted mice were treated when tumors reached a volume of 63 to 250 mm^3. Compared with the control group, treatment (a) was considered to be ineffective [tumor growth inhibition (TGI) = 38%], treatment (b) was partially effective (TGI = 79%), treatment (c) induced a TGI > 95%, and treatment (d) induced complete (clinical) regression of the tumors (TGI = 100%). In (c), mice were treated when tumors reached a volume of approximately1000 mm^3. Treatments (a and b) were considered to be ineffective, with TGI of 39% and 43%, respectively; treatment (c) induced partial response (TGI = 57%), and as before, treatment (d) induced complete (clinical) regression of the tumors (TGI = 100%). Note that the duration of in-vivo experiments was 28 and 16 days in (b and c), respectively.

prospective tumorgrafts to select the most effective chemotherapeutic drugs and/or combinations and to define an optimal tailored anticancer treatment for the corresponding patient's tumor. Such an approach is currently under way at the Johns Hopkins for patients with pancreatic ductal carcinoma [64,73].

Validation of the high predictive value of preclinical tumors treated by conventional chemotherapeutic agents for clinical efficacy constitutes a first but necessary step of model characterization. However, in our modern molecular management of malignancies, the real question concerns the predictive value of tumorgrafts to targeted therapies and their clinical efficacy. Beyond the expression of membrane receptors that are targeted by specific antibodies and/or tyrosine kinase inhibitors, such as Her2 and EGFR, specific inhibition of molecular pathways involved in oncogenesis, such as Notch, Hedgehog, and others, is a very exciting challenge for the future in clinical practice and in the preclinical setting. Of course, this is the challenge of preclinical models, the value of established tumorgrafts as predictive models for clinical results remains to be confirmed for new molecular targeted compounds under development, both in an individual or a combined treatment setting. However, one basis for such an approach is to start preclinical experiments in well-validated xenografts for conventional anticancer therapies, as this situation mimics the clinical history of patients with cancer initially treated by conventional chemotherapy and/or radiotherapy. Moreover, it is important to mention that targeted therapies could not be directed by exclusive classical 'maximal tolerated dose' criteria, as standard chemotherapeutic agents. Indeed, what is probably of high importance for targeted therapies are pharmacodynamic data that are able to state on the good reach of the target, how this targets impact the tumor responses, and how it could be improved in low-responding or refractory patients.

Ability of the model to mimic clinical situations

The last challenge to be met by human preclinical tumor models is to closely mimic clinical situations observed during the course of human cancer. Unfortunately, three main clinical situations are frequently observed: metastatic progression, relapse after obtaining a first treatment-induced CR, and primary or secondary treatment resistance. All these situations constitute pejorative events for patients with cancer, regardless of the type of cancer, as they indicate more aggressive and less sensitive disease. Identification of treatments that could be effective in metastatic, relapsed, and/or resistant disease constitute a major challenge for oncologists, researchers, and drug companies. The establishment of preclinical tumorgraft models mimicking this type of clinical situations would therefore be very useful in this approach.

Metastatic tumorgraft models

As the patient's tumor sample is derived from a primary or metastatic lesion, in-vivo transplantation is consistently performed in a single orthotopic or nonorthotopic site (generally into the interscapular fat pad of the mouse). After establishment and characterization of the model, its metastatic potential must be evaluated. The simplest way to evaluate metastatic potential is to perform a pathological examination after ethical killing of the mouse while looking for cancer cell islets in various organs, mainly the liver, lungs, bones, and brain. Under these experimental conditions without ex-vivo manipulations and specific in-vivo tumor cell inoculations, few models could have developed spontaneous metastatic lesions (Fig. 3a). In our experience of BC tumorgrafts, lung metastases were observed in 0% of transplanted mice in seven of 17 models, in 5–20% of mice in seven of 17 models, and in 80–100% of tumor-bearing mice in three models [15]. However, a major limitation of this experimental approach is the relatively short interval between the initial in-vivo transplantation and killing of the mouse, which could not be sufficient for the primary tumor to develop metastases. Other experimental approaches must therefore be developed to improve the metastatic rate of tumorgraft models as follows: (i) the first approach consists of intravenous injection of tumor cells, or possibly intracardiac administration [74], to artificially induce lung or liver metastatic colonies [69,75]. However, in these experiments, metastatic proliferation is induced by the filter formed by the liver and lung capillaries and not by molecular changes in cancer cells leading to primary tumor release, the ability to circulate and survive under the stringent conditions of circulating blood, and subsequently seed in heterotopic organs. It has not been established that this type of artificial metastatic tumor cells truly reflect the biology of metastatic tumors in patients with cancer, suggesting that their pharmacological properties may not constitute a formal and unquestionable read-out for assessment of the efficacy of antimetastatic therapies. (ii) The second experimental modality consists of surgically removing the primary tumor to allow a longer time for metastatic dissemination before ethical killing of the mouse (Fig. 3b). This approach consists of all of the biological events required in the metastatic process, but with the exception of part of the host–molecular interactions that are important actors during the metastatic process, and it appears to be more relevant for pharmacological assessment of new antimetastatic compounds. However, the possibility that surgical excision of the primary tumor leads to artificial release and circulation of cancer cells into the vascular system cannot be excluded. (iii) The third approach consists of repeated excision and in-vitro culture of metastatic lung lesions to finally obtain a more suitable metastatic disease [76]. As the establishment of these models would be a very long process, the main criticism of this approach concerns the numerous ex-vivo manipulations of cancer cells that could modify their biological and molecular characteristics.

Consequently, pharmacological assessment of these metastatic models could be influenced by these modifications. Using a similar approach, serial SC passages of lung metastases have been performed to develop a spontaneous human lung metastatic xenograft [77]. (iv) Another experimental procedure consists of orthotopic tumor implantation that may increase metastatic dissemination [74,78,79]. However, although such an experimental approach closely mimics human clinical situations and appears to be the best experimental procedure, the feasibility of orthotopic implantations constitutes a major limitation to its use. (v) Finally, subcutaneous transplantation of human bone has also been performed to induce bone metastases of PC cells [80,81].

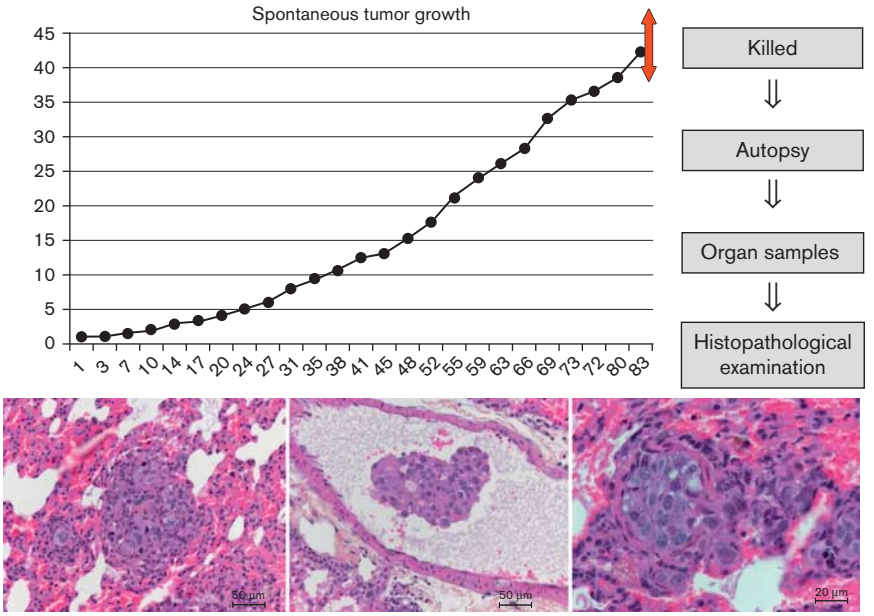
As previously suggested, pharmacological assessment of antimetastatic therapies does not only require preclinical metastatic models but also relevant metastatic sites. For instance, bone lesions are frequent in PC, but not in uveal melanoma; inversely, liver metastases are rare in PC and frequent in uveal melanoma. To closely mimic clinical situations, it is therefore important to bear in mind the natural outcome of the tumorgraft and whether or not this coincides with the natural outcome of the corresponding human cancer. Moreover, in few uncommon situations, due to growth factors that specifically act on stromal cells or the metastatic microenvironment, the primary tumor and metastases have been shown to present different responses to treatment [82].

Tumorgraft models of relapse after initial complete remission

Relapses after initial CR constitute frequent events during the course of human cancers. As no tumor disease can be detected by conventional investigations, that is, CT scan, biological markers, and other parameters; recurrences are obviously due to microscopic residual cancer cells that are able to resist first-line treatment and to survive a long time in a latency state, before subsequently growing in local and/or metastatic sites. 'Basal-like' BCs could be the paradigm of this type of situation, with a high sensitivity to initial combination chemotherapy and a very high risk of relapse [70]. This unusual outcome raises two main questions: the first concerns the treatment modalities that can be proposed to reduce the risk of recurrence after obtaining CR and the second concerns the biological features of residual cancer cells that allow them to resist and survive.

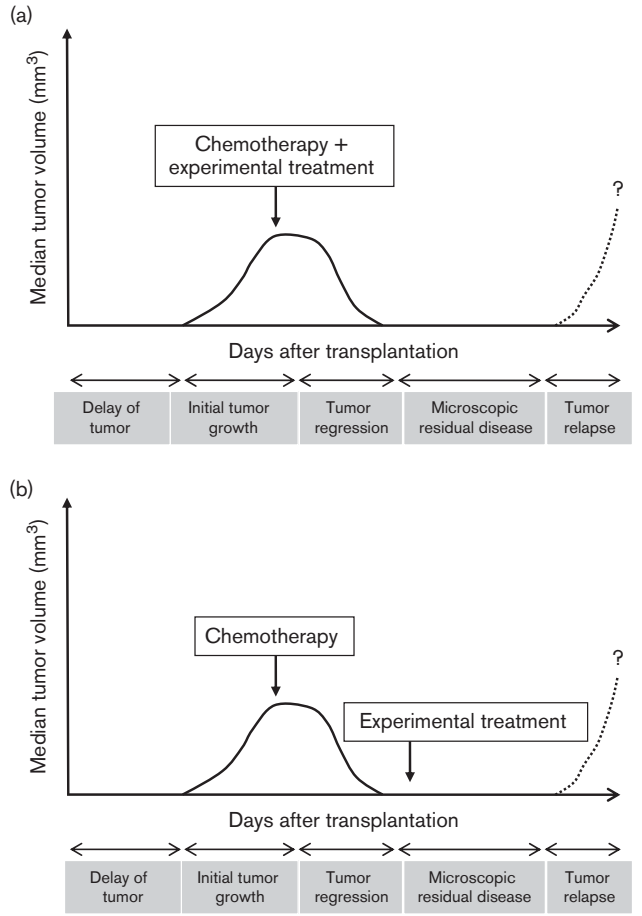
Preclinical models such as tumorgrafts often present a different sensitivity to conventional and/or innovative therapies. Some experimental treatments are able to completely inhibit in-vivo tumor growth resulting in complete disappearance of the measurable 'clinical' tumor mass. However, in the majority of pharmacological experiments, the outcome of xenografted mice after obtaining CR is rarely evaluated, and the search for residual cancer cells at the initial site of transplantation has rarely been performed. In our laboratory, we have developed various models of 'basal-like' BC tumorgraft in

Fig. 3



Spontaneous emergence of in-vivo metastatic lesions. Tumor-bearing mice were killed at the ethical limit (2500 mm^3) and lungs as well as other organs were then submitted to histopathological examination. Intraluminal and intraparenchymal lung metastases were then detected after hematoxylin and eosin staining, combining experimental treatment concomitantly with chemotherpy (a) or after complete remission induction (b).

Fig. 4



Prevention of relapse. Development of a model of 'basal-like' breast cancer tumorgraft, in which complete remission was induced after chemotherapy (doxorubicin and cyclophosphamide) with relapse constantly occurring after several weeks at the initial site of transplantation. Experimental assessments could therefore be performed with a read-out focused on relapse rate, combining experimental treatment concomitantly with chemotherapy (a) or after complete remission induction (b).

which CR is first induced by standard chemotherapy (doxorubicin and cyclophosphamide, and other agents) and which subsequently remains at the stage of microscopic residual cancer cells detected by histopathological and/or PCR analyses for a median of 8 weeks, before finally relapsing at the initial site of transplantation [83], (and unpublished data). As shown in Fig. 4, this type of tumor growth profile allows evaluation of new therapies that could be concomitantly combined with chemotherapy (a) or administered as adjuvant treatment after obtaining CR (b), with a main read-out defined as the relapse rate in both situations, as already performed with antibody-mediated CD44 targeting [83]. Detection of residual tumor cells also provides an opportunity to study these cells in various biological studies. It has been suggested that residual BC cells may contain a significant fraction of the so-called 'tumor-initiating cells' [84], as we have shown that residual cells of the BC tumorgraft were CD44-positive [83,85]. This finding could therefore constitute the third possible application of a preclinical model of relapsed tumor.

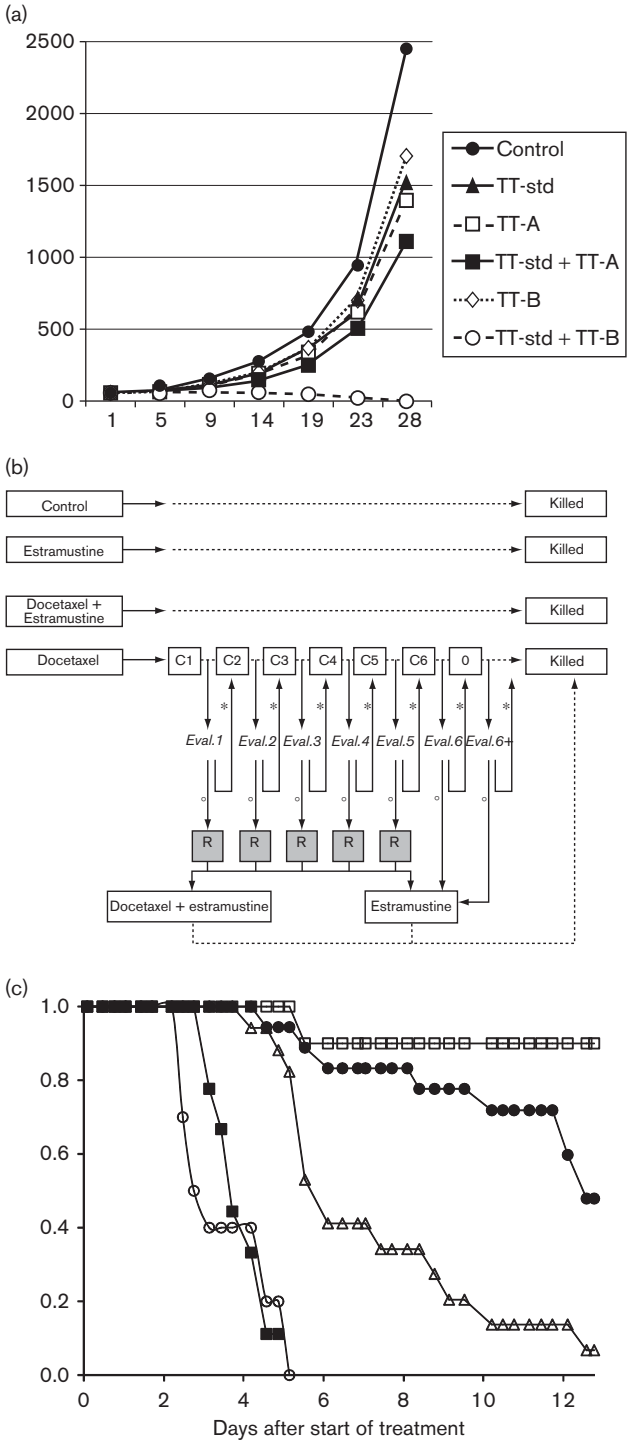
Preclinical assessment of primary or secondary treatment resistance

Various clinical situations show the impact of treatment resistance on the outcome of patients with cancer: (i) local and/or metastatic relapses occurring after the end of treatment illustrate the capacity of cancer cells to survive after antineoplastic therapies. As previously mentioned, local subclinical residual tumor cells after first-line therapy constitute primary resistant cancer cells in this context; (ii) resistance may also emerge during the course of treatment, either immediately or after initial tumor regression, during short induction treatment phases such as chemotherapy or a long maintenance phase such as hormonal therapies used, for instance, in patients with luminal BC. All these clinical situations, frequently observed by oncologists, should therefore be modeled in preclinical experiments. Tumorgrafts could be used to develop various experiments to evaluate primary and secondary treatment resistance, followed by pharmacological assessment of anticancer drug-resistance strategies. It should be stressed that the validity and predictive accuracy of this type of preclinical model of anticancer treatment resistance are strengthened by the marked histopathological, molecular, and genomic similarity of tumorgrafts and human cancers. This obviously does not apply to xenografts derived from human tumor cell lines often used for this purpose.

Various preclinical models of therapeutic resistance could be developed from primary human cancer xenografts (Fig. 5): (i) therapeutic characterization of the models by determination of response to standard treatments, leading to the constitution of nonresponding tumorgrafts that could be subsequently used to assess innovative therapy either alone or in combination with inefficient standard therapy. In the context of the development of new targeted compounds, such experiments are performed to introduce these agents as early as possible into the design of clinical trials and possibly into clinical practice. There are many examples of this type of classical approach and the relevance of their results is directly related to the accuracy of the model used. (ii) Induction of secondary resistance to continuously maintained treatment in in-vivo tumorgrafts. As this type of approach could be used for standard chemotherapy, it appears particularly relevant, in the clinical practice setting, for maintenance therapies, that is, hormonal therapy, monoclonal antibodies, and other treatments. We have generated numerous hormone-independent PC variants from the hormone-dependent PC model PAC120 [86] that have been used for molecular studies as well as pharmacological assessments on both hormone-dependent and

hormone-independent models [87–90]. (iii) Another experimental design, similar to the previous situation, is defined by longitudinal studies and repeated tumor biopsies or cancer cell cytoaspiration in each xenografted mouse, studies that are performed to define biomarkers of response and resistance. As these in-vivo experiments are based on a very simple principle, two main issues remain to be clarified. First, it has to be determined whether or not tumor biopsies or cancer cell aspirations could impact on natural in-vivo tumor growth. Second, it must be verified whether the quantity and quality of in-vivo material removed are sufficient for molecular analyses including mRNA and protein studies. (iv) The last preclinical approach is also highly relevant in terms of the patient's clinical outcome, namely treatment of progressive disease on treatment. This situation closely mimics that frequently observed in patients with cancer who have received several different courses of chemotherapy, particularly in a more pejorative metastatic setting. Such an experimental design allows the individual management of xenografted mice, each corresponding to one patient, according to the treatment response evaluated before administration of each cycle. Resistant mice are then randomized into various groups evaluating salvage treatments. We have used the hormone-dependent PAC120 model to evaluate the role of estramustine combined with docetaxel from the stage of initial tumor growth and after the emergence of docetaxel resistance (Fig. 5d and e) [90]. We have shown that, in docetaxel-refractory tumor-bearing mice, estramustine alone had a slight and short efficacy, whereas estramustine combined with docetaxel induced intense and prolonged tumor growth inhibition, suggesting that estramustine administration can be delayed and combined with docetaxel in docetaxel-refractory PCs with no negative impact on outcome.

Fig. 5



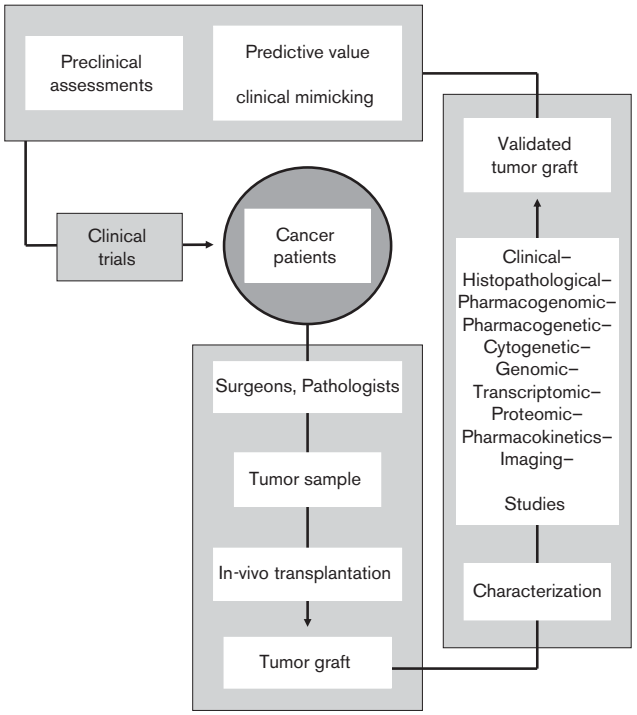
Present and future

The establishment of preclinical models of human primary tumors constitutes a long-term process requiring various collaborations to allow stabilization and characterization of tumorgrafts. This process must include

Preclinical assessment of primary or secondary treatment resistance. (a) Compared with the control group, standard treatment induced a tumor growth inhibition (TGI) of 38%. Two new compounds were evaluated, treatments (a and b), which induced TGI of 43 and 31%, respectively. All these treatments administered alone were then considered to be ineffective. The combination of standard treatment + treatment (a) induced a slight increase of TGI (55%) but was unable to reverse tumor resistance, whereas standard treatment + treatment (b) induced complete remission (TGI = 100%) and completely reversed primary tumor resistance. (b) In the human prostate cancer PAC120 model, secondary resistance to docetaxel was defined at the beginning of each next cycle ($n+1$) as an relative tumor volume$_{n+1}$/relative tumor volume$_n$ ratio of more than or equal to 2. Resistant mice were then randomized into two groups, one receiving estramustine alone, and the other treated by a combination of docetaxel + estramustine with final evaluation (Eval.) of tumor growth delay for a two-fold tumor size increase (tumor growth delay$_2$). (c) In docetaxel-refractory tumor-bearing mice, estramustine alone had a slight and short efficacy, whereas estramustine combined with docetaxel induced a marked and prolonged improvement of overall survival. Moreover, the overall survival of mice treated by docetaxel plus estramustine since day 1 or since resistance to docetaxel was not significantly different. These results therefore suggest that estramustine administration can be delayed and can be combined with docetaxel in docetaxel-refractory prostate cancers with no negative impact on outcome.

updating of biological studies, according to the type of cancer, and a clinical approach to resolve questions raised by the natural outcome of the patients. Figure 6 illustrates the various steps involved, from collection of the patient's tumor sample to validation of a relevant preclinical model. Obviously, not all experiments indicated in the figure should be considered as prerequisites for validation of the model, but definition of the various steps to be performed is an essential aspect that must be conducted jointly by clinicians, biologists, and scientists. This approach can be guided by a few simple questions: (i) what are the minimum tests required to confirm the clinical diagnosis (i.e. biological relevance), (ii) what is the natural history of the cancer and the subcategory of cancer from which tumor samples were obtained (i.e. clinical relevance), (iii) which clinical and biological properties of the model are useful for efficient pharmacological experiments (i.e. clinical and biological relevance), (iv) what are the standard treatment regimens used for the disease (i.e. clinical relevance), and (v) what are the factors influencing the choice of treatment (i.e. biological relevance)? All steps of validation for a relevant pharmacological assessment of a preclinical model are indicated in Fig. 6.

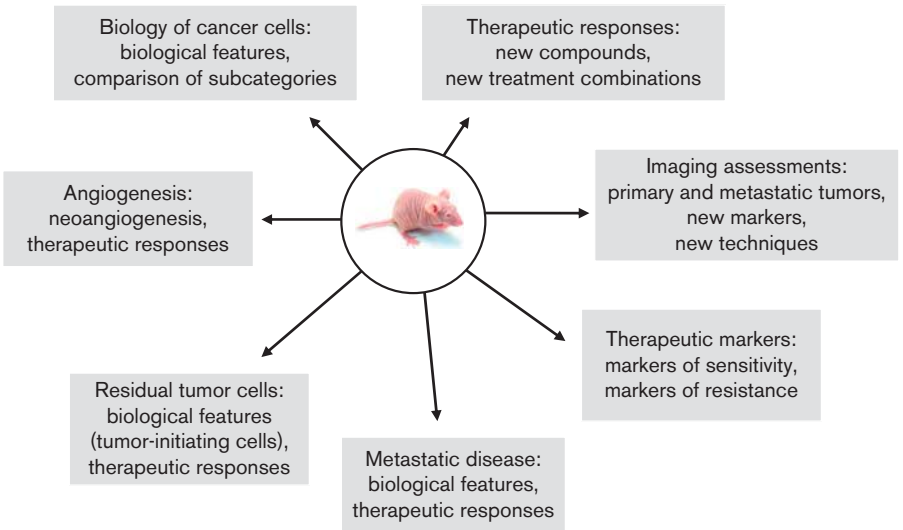
Fig. 6



Various steps from the patient's tumor to preclinical-based clinical trials. This figure describes the overall process of validation of the model, including biological and therapeutic experiments.

Finally, a better knowledge of oncogenesis on the basis of therapeutic or nontherapeutic approaches will determine the future of tumorgrafts. The establishment of models that closely mimic clinical situations in terms of biological features, in-vivo natural history, and response to treatment will provide the necessary experimental conditions to evaluate fundamental issues in cancer, that is, markers of response and resistance identifying the mechanisms of various molecular pathways, biology of residual cancer cells and tumor-initiating cells, characteristics of metastasis, angiogenesis, and tumor–stroma interactions. The

Fig. 7



Various applications of tumorgraft models. This figure describes all of the research applications that could be developed on a well-validated preclinical tumorgraft.

large availability of tumor material and the possibility to repeat experiments several times are both factors in favor of the use of biological studies in human cancers. Moreover, in some situations, complementary analyses performed on fresh, fixed, and/or frozen tumors are a way to increase the accuracy of preclinical results. A continuous 'to and fro' between these two tumor samples would increase the validation and generation of new mechanistic hypotheses, therefore leading to new knowledge on oncogenesis. All of the potential applications of tumorgrafts are presented in Fig. 7. The establishment of preclinical models of primary human tumors is therefore a useful tool for oncologists, cancer biologists, and research scientists for whom the final objective can be summarized as follows: 'to ensure optimal management of human cancer patients'.

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Conflicts of interest

There are no conflicts of interest.

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