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# The Biology of Malignant Mesothelioma and the Relevance of Preclinical Models

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Malignant mesothelioma (MM), especially its more frequent form, malignant pleural mesothelioma (MPM), is a devastating thoracic cancer with limited therapeutic options. Recently, clinical trials that used immunotherapy strategies have yielded promising results, but the benefits are restricted to a limited number of patients. To develop new therapeutic strategies and define predictors of treatment response to existing therapy, better knowledge of the cellular and molecular mechanisms of MM tumors and sound preclinical models are needed. This review aims to provide an overview of our present knowledge and issues on both subjects. MM shows a complex pattern of molecular changes, including genetic, chromosomic, and epigenetic alterations. MM is also a heterogeneous cancer. The recently described molecular classifications for MPM could better consider inter-tumor heterogeneity, while histo-molecular gradients are an interesting way to consider both intra- and inter-tumor heterogeneities. Classical preclinical models are based on use of MM cell lines in culture or implanted in rodents, i.e., xenografts in immunosuppressed mice or isografts in syngeneic rodents to assess the anti-tumor immune response. Recent developments are tumoroids, patient-derived xenografts (PDX), xenografts in humanized mice, and genetically modified mice (GEM) that carry mutations identified in human MM tumor cells. Multicellular tumor spheroids are an interesting *in vitro* model to reduce animal experimentation; they are more accessible than tumoroids. They could be relevant, especially if they are co-cultured with stromal and immune cells to partially reproduce the human microenvironment. Even if preclinical models have allowed for major advances, they show several limitations: (i) the anatomical and biological tumor microenvironments are incompletely reproduced; (ii) the intra-tumor heterogeneity and immunological contexts are not fully reconstructed; and (iii) the inter-tumor heterogeneity is insufficiently considered. Given that these limitations vary according to the models, preclinical models must be carefully selected depending on the objectives of the experiments. New approaches, such as organ-on-a-chip technologies or *in silico* biological systems, should be explored in MM research. More pertinent cell models, based on our knowledge on mesothelial carcinogenesis and considering MM heterogeneity, need to be developed. These endeavors are mandatory to implement efficient precision medicine for MM.

**Keywords: thoracic cancer, mesothelioma, molecular characteristics, tumor heterogeneity, preclinical models, cell models, animal models**

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## INTRODUCTION

The therapeutic options for malignant mesothelioma (MM) are limited, especially for the most common form of mesothelioma, malignant pleural mesothelioma (MPM). Current MPM chemotherapy is based on intravenous injections of pemetrexed (PMTX) and cisplatin or carboplatin. Recently, this basic treatment has been improved by the addition of vascular endothelial growth factor (VEGF) antibodies (bevacizumab), where the overall survival of patients receiving PMTX, cisplatin, and bevacizumab was significantly enhanced (MAPS study) (1). Furthermore, immunotherapy-based strategies are currently becoming attractive therapeutic options, and several clinical trials have recently been performed. A phase II study using monoclonal antibodies against cytotoxic T-lymphocyte antigen 4 (CTLA4; tremelimumab) in patients showing progression of the disease after first-line treatment yielded encouraging results, but it was performed in a small number of patients (2). Another phase II study (DETERMINE) investigated the effect of tremelimumab in patients whose disease had progressed after one or two systemic treatments. There were no benefits, but the safety profile was acceptable (3). A more recent phase 2 study (IFCT-1501 MAPS2) reported the use of immune control checkpoint inhibitors, programmed cell death protein 1 (PD-1; nivolumab) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4; ipilimumab), alone or in combination. The results showed objective anti-tumor responses and a significant increase in survival without progression and global survival (4). Clinical trials for cell-based immunotherapy using dendritic cells or chimeric antigen receptor T (CAR-T) cells have also yielded promising results (5–10).

In the future, new clinical trials will be developed that utilize novel anti-cancer compounds or immunological modulators in association with chemotherapies or in combination with immunological approaches. The efficiency of current treatments are dependent on the integrity of metabolic pathways and DNA repair mechanisms that account for resistance mechanisms. Overall, therapy improvements require better knowledge of the state of the cell regulatory pathways. In addition, immunotherapies need sound knowledge about the immunological status of the tumor. To date, molecular data are not ordinarily used to assist therapeutic decisions, and thus there is an urgent need for their use in translational medicine. To reach these goals, two different fields must be investigated: (i) the cellular and molecular status of MM tumors, regarding mutations, alterations in regulatory pathways, and the microenvironment landscape, and (ii) the methodology of preclinical assays to soundly test specific anti-tumor agents. The aim of this review is to provide an update on our present knowledge and issues on these subjects and to provide perspectives for advancements in MM treatment.

**Abbreviations:** 2D, two dimensional; 3D, three dimensional; CDX, cell-derived xenograft; CGH, comparative genomic hybridization; GEM, genetically modified mice; hom, homozygous; htz, heterozygous; IPl, intra-pleural; IPe, intra-peritoneal; luc, firefly luciferase; MCTS, multicellular tumor spheroids; MM, malignant mesothelioma; MPM, malignant pleural mesothelioma; MPeM, malignant peritoneal mesothelioma; MRI, magnetic resonance imaging; NGS, next generation sequencing; NSG, NOD-scid IL2Rγnull; PDX, patient-derived xenografts; PMTX, pemetrexed; SC, subcutaneous; SNP, single nucleotide polymorphism; SPECT, single photon emission computed tomography; TSG, tumor suppressor genes.

## THE BIOLOGY OF MALIGNANT MESOTHELIOMA

Malignant mesothelioma are heterogeneous tumors that show a complex pattern of molecular changes, including genetic, chromosomic, and epigenetic alterations, all of which should be considered to model this pathology. Of all the MM types defined by tumor location, MPM has the best described molecular alterations and heterogeneity, and thus we will focus on it. Notably, recent integrative multi-omics analysis as well as next generation sequencing (NGS) studies on malignant peritoneal mesothelioma (MPeM) showed similarities to MPM in terms of molecular alterations, even though some alterations, such as *ALK* rearrangement, are only found in MPeM (11–13).

### Molecular Alterations

Recent NGS studies identified a low mutation burden in MPM compared to other adult solid tumors (14). However, this mutation burden could be underestimated by classical NGS analyses, which focus on the detection of changes at the nucleotide level. Early karyotyping analyses and molecular cytogenetic techniques, such as comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays, showed that MPM is characterized by numerous chromosomal abnormalities, including abundant numeric and structural chromosome changes and recurrent alterations in specific chromosome regions (15). More recently, a combination of high-density array-CGH with targeted NGS demonstrated the presence of chromothripsis in the 3p21 region, which includes the *BAP1* gene (16). Chromothripsis and also chromoplexy were confirmed on several other chromosome regions in MPM using mate-pair sequencing (17). These numerous inter- or intra-chromosomal rearrangements may result in the disruption of tumor suppressor genes (TSG) as well as the amplification of oncogenes or fusion genes that can drive carcinogenesis.

The mutated genes in MPM are essentially TSG that are inactivated by several mechanisms, including single nucleotide variants, copy number losses, gene fusions, and splicing alterations (14, 18). The only recurrent oncogenic mutation was identified in the promoter of *TERT*, which encodes telomerase, the essential enzyme that maintains the length of the telomeres (19). The most frequently altered TSG are *CDKN2A*, *BAP1*, and *NF2*, and to a lesser extent *TP53*, *SETD2*, and *LATS2*. All of the other mutated genes show <3% somatic mutation (14, 18). Germline mutations that predispose to MPM were first identified in *BAP1*, but two recent studies also highlighted germline mutations in several other genes that are less common than in *BAP1*. They are mainly involved in cell-cycle, chromatin regulation and DNA repair (20–24). Up to 7% of MPM patients may have germline mutations, but experimental validations

are needed to confirm that some of these genes are MPM susceptibility genes (like *BAP1*).

The epigenomic landscape of MPM has also been investigated, albeit to a lesser extent. A microarray-based methylome analysis demonstrated that MPM has specific patterns of gene methylation compared to normal pleura or other tumors (25, 26). The contribution of DNA methylation to mesothelial carcinogenesis has been clearly established, notably by the downregulation of TSG expression (27). The mechanisms for epigenetic regulation in MPM were principally studied in the context of *BAP1* inactivation; they highlighted the role of polycomb repressive complex 2 (PRC2) and histone methyltransferase (28). Other studies also emphasized the involvement of non-coding RNA such as micro-RNA (miRNA) or long non-coding RNA (lncRNA), both of which are deregulated in MPM, in carcinogenesis (29–31).

Altogether, these molecular alterations lead to changes in gene expression and deregulation of several biomolecular pathways, including signaling pathways such as Hippo or the PI3K/AKT/mTOR pathways, the cell cycle and apoptosis, among others (32). The implication for therapy from all these molecular changes has been recently reviewed (33).

## Mesothelioma Heterogeneity

Like most adult solid tumors, MM is a heterogeneous cancer with high variability among patients. Hence, the development of experimental models must consider this heterogeneity. Histology defines three major types of MM: epithelioid, the most frequent histological subtype; sarcomatoid, with the worst prognosis; and biphasic, which is a mixture of the two previous morphologies. Histological subtypes within these three types have been defined (34). The histological classification only partially captures the tumor heterogeneity observed at both the molecular and clinical levels (35). Large-scale omics and NGS studies have demonstrated MPM heterogeneity at the molecular level that goes beyond the histological classification (14, 18, 36). The first MPM molecular classification, related to histological types and survival, proposed two tumor subtypes by clustering transcriptomic data (36). A new subtype with a poor prognosis and characterized by a double mutation in the TSG *NF2* and *LATS2*, both of which are involved in the Hippo signaling pathway, was identified by coupling genetic and transcriptomic analysis (37). Other studies have proposed classifications into four subtypes that are also related to prognosis and partially to genetic alterations (14, 18, 38). Interestingly, a meta-analysis that compared the subtypes obtained by clustering from several transcriptomic data sets showed that only the most extreme subtypes, which represent the "pure" epithelioid and sarcomatoid phenotypes, are found in all datasets. These findings suggest that intermediate subtypes might only reflect divisions of a continuum (38, 39). Based on these results, histo-molecular gradients obtained by a signal deconvolution method on transcriptomic data were proposed to consider MPM inter-tumor heterogeneity as well as intra-tumor heterogeneity. These histo-molecular gradients determine the variable proportion of epithelioid and sarcomatoid tumor cell contingents in tumor samples. They also have a strong prognostic value and may be of interest for guiding therapeutic strategies (38, 39). Another recent publication further sustained that MPM heterogeneity is better described by a continuum (40).

Intra-tumor heterogeneity is still partially described in MPM, in part due to the use of omics approaches only in bulk tumor samples. MPM is likely a polyclonal tumor that comprises multiple subclones with variable cellular prevalence (41, 42). To better define the polyclonal tumor origin and understand the tumor evolution of mesothelioma, further studies are required in a larger number of tumor samples. Several studies also highlighted the presence of cancer stem cells in MPM (35). In MPM, heterogeneity is not limited to tumor cells; the tumor microenvironment is also distinct from one patient to another in terms of type and number of stromal and immune cells that infiltrate the tumors (43). Immune signatures are linked to the patients' outcome (44). Spatial heterogeneity of the somatic mutations of cancer cells, as well as the immune microenvironment, was highlighted by studying tumor samples at different anatomic sites (45). In this complex context, the use of the emergent "single cell" approaches will be helpful in providing an accurate characterization of tumor and stromal cell heterogeneity and should contribute to a breakthrough in knowledge about intra-tumor heterogeneity. Besides the inter- and intra-tumor heterogeneity of tumor cells, the evolutionary features of tumors need to be considered to establish a classification that is clinically relevant (46).

# PRECLINICAL MODELS

In this section, we will focus on preclinical models that are useful for chemotherapy, targeted therapy, or immunotherapy rather than for surgery or radiotherapy, even though those therapies have a place in the treatment of patients. The efficiency of anti-cancer compounds to treat MM patients has been tested using large variety of so-called preclinical MM models. These systems are based on use of human or mammalian MM samples, i.e., xenografts in immunosuppressed mice or isografts in syngeneic rodents. Multiple combinations have been developed based on the nature of the malignant sample (cells or tumor tissue), the recipient (rats or mice), the anti-cancer agent (anti-cancer drug, lytic virus, therapeutic cells sur as dendritic or CAR-T cells, etc.), the agent vector (if any), the method to implant tumor cells, and the analytical method. These models do not exactly reproduce human MM, but they are surrogates for a proof of concept. Preclinical model options are synthetized in **Figures 1**, **2**, and the main points are described below:

(i) Samples and recipients (**Figure 1**): Several MM samples are used for preclinical studies. Tumor fragments, pleural liquid, and ascites can be collected from patients. Commercial MM cell lines are available from different companies, but primary MM cell lines are a better model, as extensively discussed in the *in vitro* models section (see below). Cell models in culture mostly comprise two dimensional (2D) MM cells or three dimensional (3D) multicellular tumor spheroids (MCTS). These cell models are generally monoculture, but new developments include the introduction of stromal and immune cells to better

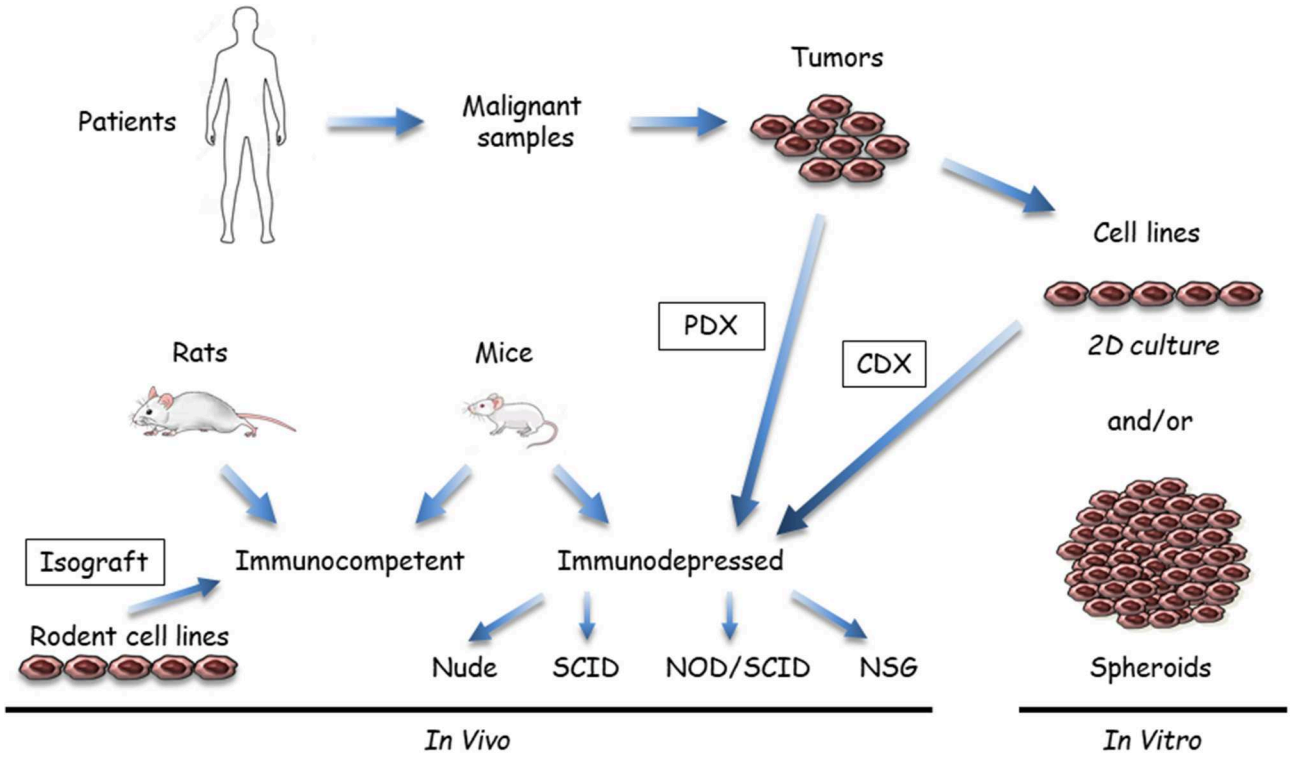


**FIGURE 1 |** Available malignant mesothelioma (MM) preclinical models that use tumor samples or cell lines. Malignant samples are tumor fragments or more often MM cells obtained after tissue dissociation; these samples are used *in vitro* or transplanted/inoculated in immunosuppressed or immuno-compatible rodents, almost exclusively mice. *In vitro*, two-dimensional (2D) cultures or three dimensional (3D) spheroids can be grown from tumor tissue samples. *In vivo*, MM tumor cells in culture are inoculated either subcutaneously or orthotopically (intracavitary, in the pleura or peritoneum) to generate cell-derived xenograft (CDX) or isograft. Tumor fragments can be also engrafted into immunosuppressed mice (patient-derived xenograft [PDX]). Immunocompetent models mainly comprise syngeneic rodent models. Human immunocompetent models can be obtained using NOD-*scid IL2Rγ*$^{null}$ (NSG) mice.

recapitulate the tumor microenvironment. *In vivo* models are based on the injection of MM cells in subcutaneous (SC) or orthotopic (intrapleural [IPl] or intraperitoneal [IPe]) sites in relevant rodents, mainly mice. Fresh MM tissue samples can be also grown as tumoroids (tumor-derived organoids) in culture or xenografted in immunosuppressed mice as patient-derived xenografts (PDX). Regarding the heterogeneity of human MM, it is important to work with well-characterized MM, particularly when drugs have been designed to target a single protein or a specific pathway. With our developing knowledge of the MM biology, it appears that multiple samples would have to be used. Furthermore, MM classification according to data arising from multi-omic studies (12, 14, 18, 36, 38) might help to define key alterations representative of molecular subtypes of MM and limit the studies on representative samples.

(ii) Anti-cancer compounds (**Figure 2**): These compounds are intended for chemotherapy, target therapy, immunotherapy, gene therapy, or oncovirotherapy because MM is a compartmentalized tumor with accessibility for *in vivo* local delivery (47, 48). They are used alone or in combination with other compounds, and as a single molecule or vectorized. Preclinical studies on the chemotherapeutic agent PMTX illustrated this diversity. The effects of PMTX have been investigated in association with several anti-tumor agents (anti-tubulin, gemcitabine, cisplatin, anti-thymidylate synthase, RNA interference [RNAi] embedded in liposomes, miRNA expressed in adenovirus vector, etc.) to determine a potential synergistic effect (49–55). Liposomal PMTX formulations have been tested in an orthotopic mouse model (56). Due to the diversity of the assays, it is difficult to compare their predictability.

(iii) Analytical methods (**Figure 2**): The endpoints for *in vitro* assays comprise the determination of cell proliferation, cell death, motility and invasive properties, and spheroid state (morphology and volume). *In vivo* tumor analyses involve macroscopic and microscopic observations and evaluation of immune infiltration and angiogenesis. The key point is to monitor tumor evolution, especially for orthotropic tumor grafts. Different analytical methods have been developed for *in situ* tumor visualization. Firefly luciferase (*luc*)-engineered cells can be detected by a non-invasive bioluminescence imaging method, as in rats injected with *luc*-MM cells in the pleural cavity (57). However, data have shown that magnetic resonance imaging (MRI) is a more reliable method for MPM tumor burden measurement compared to bioluminescence (57). Computed tomography scanning may be also of interest, as shown with a lung cancer cell line in mice (58). Tumor lesions and the localization of

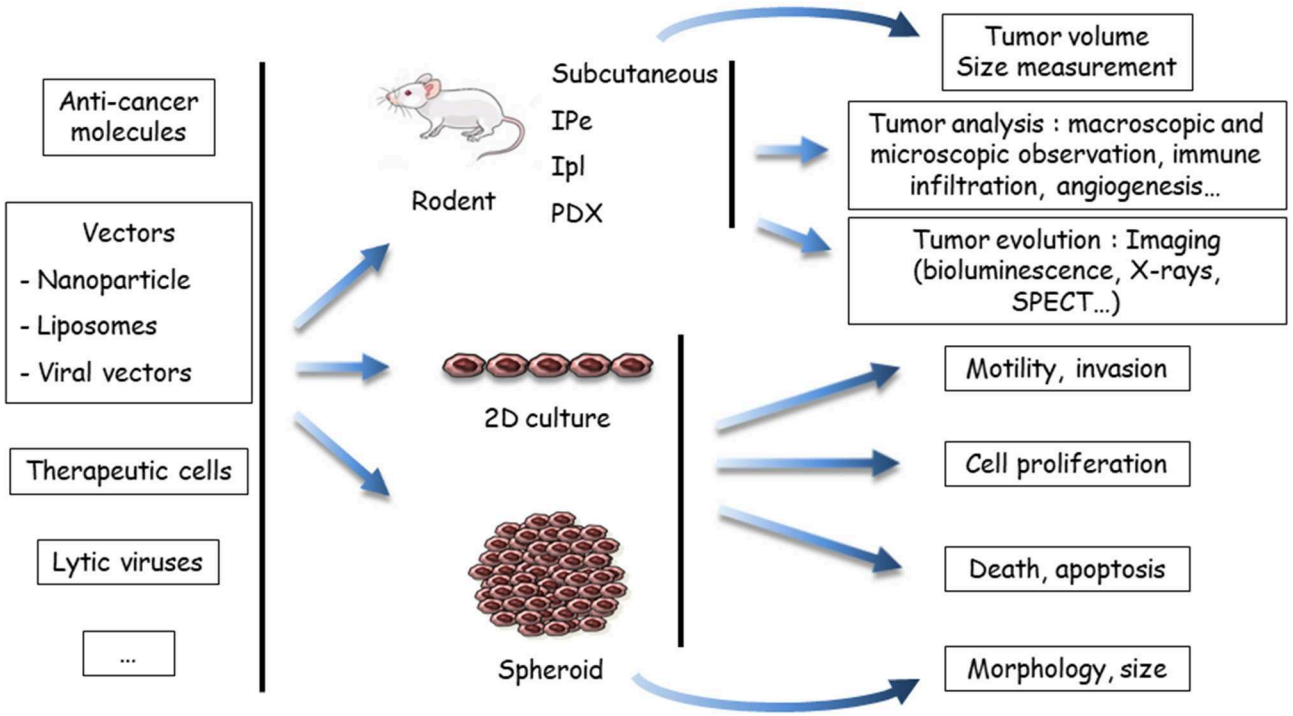


**FIGURE 2 |** Evaluation of treatments using preclinical models of malignant mesothelioma (MM). Anti-cancer agents are delivered to the MM models, either in a native, or vectorized form (in nanoparticles, liposomes, or viruses). MM cells may be labeled or engineered to allow one to determine tumor growth by imaging methods, mostly bioluminescence. To determine the host response, the analytical method depends on the MM model. *In vivo* endpoints comprise measuring the tumor volume, quantifying and identifying tumor cells in the pleural effusion or ascites (if produced), or measuring others parameters (angiogenesis, immune infiltration, etc.). *In vitro*, the endpoints are cell viability, type of death, proliferation, motility, invasion, morphology, and volume (spheroids). Both cell and mouse models have been applied to test the efficiency of drugs in mono- or multimodality chemotherapies, immunotherapies and target therapies, oncovirotherapies, or cell and gene therapies. SPECT, single photon emission computed tomography; IPe, intra-pleural; Ipl: intra-peritoneal; PDX, patient-derived xenograft.

epidermal growth factor receptor (HER) were visualized with single photon emission computed tomography (SPECT) and MRI in an orthotopic MM model with radiolabeled specific antibodies (59). Bioluminescence of *luc*-expressed MM remains the most common strategy to monitor tumor development in orthotopic models. These *in vivo* imaging methods require specific equipment and facilities and, for bioluminescence detection, the genetic modification of tumor cells with the *luc* gene. The introduction of an exogenous gene might have an impact on cell mechanisms and immune response.

In the following subsections, we detail the present and ongoing models, with a focus on their interest, limitations, and impacts to assess emerging therapies. The advantages and limitations of the mesothelioma preclinical models are presented in **Table 1**.

## *In vitro* Models

MM cell lines have been widely used to study MM pathogenesis and evaluate the activity of numerous anti-cancer agents. The first MM cell lines were established in 1982 from the abdominal fluid of a patient (60). In 1987, a MM cell line was established from a surgical sample of malignant pleura, namely H-Meso-1 (61). Since that study, numerous cell lines have been established from samples of patients by different groups to constitute local biocollection. Some of these collections have been extensively characterized (19, 36, 37, 62–66), as well as 21 cell lines in the Genomics of Drug Sensitivity in Cancer (GDSC) database (https://www.cancerrxgene.org/celllines). MPM cell lines present common characteristics with regard to tumors and might lead to the identification of new biomarkers (62, 67, 68). One study discussed the limits of these cell models. The authors found strong molecular differences between primary and commercial cell lines (67), mainly due to a high number of divisions after their establishment, and thus an increased risk of new karyotypic changes. These models remain interesting for screening and preliminary investigations. Primary tumor cells represent an intriguing alternative because they share similar molecular characteristic with the primary tumor, even though they show a reduction of subclonal diversity (15, 42, 67). However, the necessity to perform studies before 6–10 passages limits the number of experiments. The most appropriate strategy would probably be to conduct large screening studies on cancer cell lines and then confirm the findings with primary cancer cells. The results obtained with cell lines should be confirmed on samples from patients (if applicable).

**TABLE 1 |** Advantages and limitations of the different models of mesothelioma.

| Model | Advantages | Limits |
|---|---|---|
| Monolayer cells | Easy to obtain<br>Suitable for high-throughput screening | Clonal selection in culture<br>Response to therapies is poorly representative<br>No microenvironment |
| Multicellular tumor spheroids (MCTS) | Easy to obtain<br>Three-dimensional structure constraints<br>Suitable for high-throughput screening<br>Common features with tumors *in situ* | Not heterogeneous<br>Partial and artificial microenvironment |
| Cell-derived xenograft (CDX) | Source of tumor cells easy to obtain (cell lines in culture)<br>Useful for evaluation of targeted strategies | Not heterogeneous<br>No microenvironment<br>Immunodeficient context<br>Response to therapies is poorly representative |
| Patient-derived xenograft (PDX) | Tumors with characteristics of the of patients (heterogeneity, microenvironment)<br>Representative response to therapies | Availability of the tumor<br>Time to obtain the tumor<br>Not suitable for immunotherapy evaluation |
| Humanized model (NSG) | Human immune system<br>Evaluation of immunotherapy possible | Time to obtain tumor<br>Cost<br>Graft vs. host response |

## *In vivo* Models

The *in vivo* models that use wild type rodents and genetically modified mice (GEM) are summarized in **Figure 3**. GEM have been generated to obtain "spontaneous" MM, without exposure to asbestos fibers, by heterozygous (htz) or homozygous (hom) conditional mutation of *Ink4a* and/or *Nf2* and/or *Trp53,* or by IPl/IPe injection of Ad*Cre* to mimic the human condition (69). In this system, the rate of MPM is dependent on the type of inactivated genes; high rates occur with at least two hom genes, including *Trp53*. Survival generally exceeds 30–50 weeks, although shorter survivals occur in a few situations with hom/hom combinations (70). Similar results were recently reported by inactivating *Ink4a*, *Nf2,* and *Bap1* (71). The generated MM express a similar morphology to human MM, with a proportion of each histological type depending on the modified genes.

MM have also been generated in several types of GEM exposed to asbestos fibers IPe injections. Experimental cancers induced in animals by the responsible carcinogen better reflect the natural history of these cancers. They are particularly relevant for the coupled asbestos-MM condition, given that the large majority of human MM cases are linked to asbestos exposure. With regard to conditional mutant mice, it takes several months to more than 1 year before the development of a MM. While the morphological features are reproduced, the sarcomatoid MM subtype most frequently forms, contrary to what is observed in humans (70).

These sophisticated models have been mainly used for mechanistic purposes, with a focus on the molecular mechanism of MM formation or mechanism of action of asbestos fibers. According to our knowledge, GEM with mutated genes that are relevant to human MM have not been used to test the effects of drugs. However, models of colorectal, non-small-cell lung, and pancreatic cancers have been used to predict therapeutic responses (72, 73). Although these models are physiologically different from humans, GEM mice form tumors that carry relevant gene changes, show histological similarities, and should allow one to perform tests in an immunocompetent environment. However, there are several biological and technical pitfalls. For instance, the tumor evolution can differ among mice, with the possible occurrence of metastases, other types of tumors may be generated, and the physiological differences between mice and human may bias the predictive value of the assays. Otherwise, the complexity of these models makes it difficult to produce homogeneous data from a rather small number of mice, to detect the tumor without autopsy, to follow its evolution, and to determine the right time of its development to establish the planed protocol.

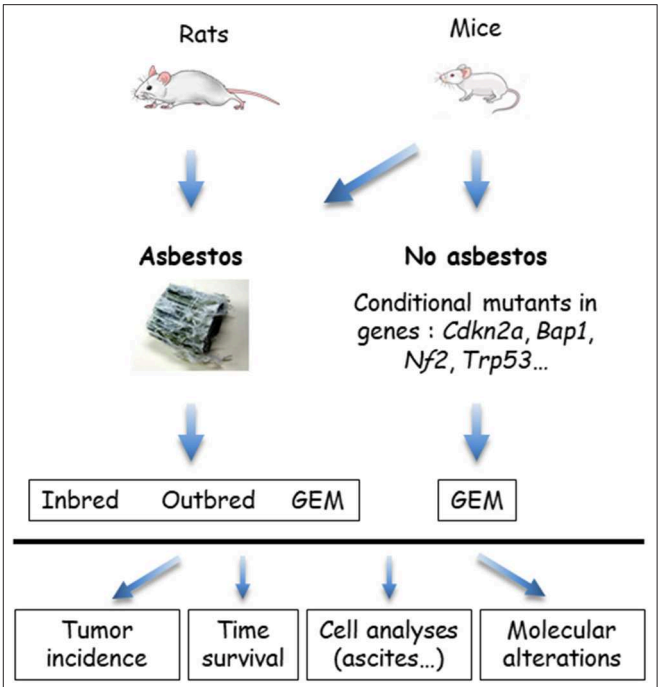


**FIGURE 3 |** *In vivo* models of malignant mesothelioma (MM) in asbestos-treated rodents and genetically modified mice (GEM). MM can be obtained from wild type (WT) rats or mice, exposed to asbestos, or GEM. Asbestos-induced MM models are generated by the injection of asbestos fibers intracavitary, in the pleura or peritoneum, mostly peritoneal in mice. Conditional mutant mice are obtained by engineering the major genes altered in MM. Asbestos-recipient animals and GEM may be investigated for tumor incidence, survival, quantitative and qualitative analyses of cells in ascites or pleural fluid, and molecular alterations in tumors.

## Specific *In vivo* Models for Immune Therapies

Recent successes were obtained with the use of immune checkpoint inhibitors in clinical trials (2, 4, 74–76). However, the response rate remains limited and, therefore, the objectives are now to extend the benefit of these approaches to a large number of patients. Preclinical studies performed in appropriate *in vivo* models are mandatory to obtain relevant results and achieve

**TABLE 2 |** The main immunocompetent rodent models of mesothelioma.

| Rodent strain | Cell lines | References |
|---|---|---|
| C57Bl/6 mice | AK7 | (77) |
| | AE17 | (78) |
| Balb/C mice | AB1 | (79) |
| | AB12 | |
| CBA/J mice | AC29 | (79) |
| Fischer F344 rats | IL45 | (80) |
| | M5-T2, F5-T1, F4-T2, M5-T1 | (81) (82) |

this objective. The first criterion is the presence of a completely functional immune system, a factor that excludes xenograft models that use human tumor cells. Several immunocompetent models of MM have been developed in rodents using cell lines obtained after inoculation of asbestos fibers in the peritoneal cavity (**Table 2**).

C57Bl/6 mice models with murine MM cell lines have been extensively used to evaluate immunotherapeutic approaches. The most utilized cell lines are AE17 and AK7 (77, 78). These cells have been modified, including exogenous expression of Ova as a neo-antigen, to increase their immunogenicity and evaluate a strategy to improve the anti-tumor immune response (78). Models of MM on the Balb/C genetic background, using AB1 and AB12 cells lines, are also available. CBA/J mice injected with AC29 cells can also be used as an immunocompetent model of MM; however, they have been exploited less than the other previously cited models (79). The main injection route to induce MM is IPe. Immunocompetent IPl models of MM have also been developed, but they are not frequently used due to the procedure required to access to pleural space. In both cases, tumor development is monitored by the previously mentioned imaging methods. SC injections are also used to overcome practical concerns, such as measurement of the tumor size and intra-tumor injection of therapeutics, but SC location is far from the pathophysiological context.

A MM model in Fischer F344 rats following IPl injection of IL45 cell line has also been described (80). With regard to rodent models, IL45 cells expressing *luc* was used to improve the monitoring of tumor development (83). Recently, models of MPeM in Fischer F344 rats have been developed (81). This effort generated several MM cell lines with distinct aggressiveness. Depending on the cell lines used, the immune infiltrate was different (with or without lymphocyte and/or macrophage infiltration of the tumors) (82). Therefore, the efficacy of immunotherapy approaches could be evaluated using this model in the appropriate immune context.

## Xenograft PDX and Humanized Models

The previously mentioned rodent models allow one to evaluate therapeutic strategies in a living organism with several constraints: elimination, diffusion in the tissue, bio-distribution, and toxicity. These models were particularly used for the evaluation of target therapies using inhibitors of histone deacetylases or signal pathways such as Hippo or focal adhesion kinase (FAK) (84–86), anti-mesothelin or anti-podoplanin antibodies (87–92), or CAR-T cells (5, 93, 94). However, they showed major limitations: (i) differences compared to humans in the immune system and metabolism of chemotherapeutic agents for syngeneic rodent models; and (ii) the use of a human cell line to induce tumors, which does not reflect human intra-tumor heterogeneity, in the context of immunodeficient mice (xenografts). In order to improve the relevance of rodent models, PDX models were developed and they implies implanting a tumor fragment from a patient into an immunodeficient mouse. For MM, the implantation was only heterotopic. Mouse strains with different levels of immunodeficiency can be used depending on the objective of the experiment (Nude, SCID, NOD-SCID, or NOD-*scid IL2Rγ*$^{null}$ [NSG]) (95). The first description of PDX models of MM was in 1980 (96). Tumors from three patients were transplanted into nude mice but only two tumors grew. Recently, SC implantation of tumors from 50 patients with MPM was evaluated in nude mice (97). This methodology maintains the heterogeneity of the tumor and its microenvironment at least during the first generation. However, the limitations include (i) a high proportion (60%) of MPM do not grow as PDX; (ii) the tumor microenvironment is replaced by murine cells over generations; and (iii) the immune context is modified, which is not suitable for evaluation of immunotherapeutic strategies (95, 97). PDX also requires access to tumor samples, which is not easy in the case of a relatively rare cancer as MM.

The use of a humanized mouse model of MM might be a good alternative to study the anti-tumor immune response. In these models, the mouse immune system is replaced by a human immune system. NSG mice are used for this research; they are deficient in the interleukin 2 receptor gamma subunit (IL-2Rγ) that is involved in differentiation and function of many hematopoietic cells (98). This feature confers a great advantage to study immunotherapy strategies in an environment that closely resembles human patients. However, these models present some limits, including the cost, the time to obtain NSG mice reconstituted with a human immune system, the risk of an incomplete differentiation of haematopoietic stem cells, and the graft vs. host reaction, which could limit the duration of the experiments.

## Spheroid Models

In order to overcome some defaults of the existing *in vivo* models, 3D tumor spheroids, positioned between 2D cell culture and animal models, have been developed (99). MCTS involves culturing tumor cells in non-adherent conditions to obtain well-rounded cellular structures after 48–72 h. This culture mode has been applied to MM cell lines. The 3D organization of cells induces major changes in gene expression compared to 2D culture (100, 101). Indeed, some pathways involved in resistance to cell death are differentially regulated in monolayer and MCTS, and thus these models better mimic resistance to treatment compared with monolayer cells (64, 102–106). These models also reproduce the diffusion constraint of therapeutic molecules, such as antibodies or nanovectors (106–109). The 3D structures also share common features with tumors from patients (101). This aspect has been notably demonstrated in the field of autophagy (110–112). Indeed, resistance to treatment is associated with autophagy in MCTS and tumors *in situ*.

The main weakness of the current 3D models is the absence of cells from the microenvironment. An alternative to MCTS is the use of tumoroids, which include tumor cells and infiltrated cells (99, 113). However, these models require access to fresh surgical MM samples. Co-cultures serve as an alternative. MCTS of non-small-cell lung cancer, pancreatic, and breast cancer tumor cells supplemented with fibroblasts and/or macrophages have been described (114–116). The tumor-associated macrophages (TAM) obtained in these models present similar characteristics to those observed in tumors, namely increased resistance to treatment and an improved cytokine environment. These aspects are crucial for immunology studies. MCTS that include different cell types might constitute interesting tools for preliminary studies. They are achieved by combining stromal and immune cells issued from cell lines or isolated from different donors. However, although their relevance needs to be confirmed, MCTS reproduce a partial human microenvironment that is completely absent from cell-derived xenograft.

## CONCLUSION

Multiple classical preclinical models of cancer have been applied, and new ones are under development, to test the potential effect of anti-cancer drugs on human MM. Each available model has benefits and limits (**Table 1**), and they must be selected depending on the objectives of the experiment. Overall, these models incompletely reproduce human MM, given that they do not consider the anatomical or biological tumor microenvironment or the intra-tumor heterogeneity of the tumors. The immunological context is not fully reconstructed, even with humanized mice. Three-dimensional spheroid cultures that have been developed as *in vitro* systems, and co-cultures with immune and stromal cells should be considered to improve the relevance of these models. Inter-tumor heterogeneity is also insufficiently studied because most models proceed with MM cell lines or tumors not always characterized at the molecular level, especially concerning the mutation burden and chromosomal abnormalities of the tumor cells. These models remain surrogates; however, they are of paramount importance in translational research and this encourage new developments to improve their predictability. Among recent developments are PDX models and the generation of GEM that carry mutations identified in human MM tumor cells. These approaches may be useful, but PDX and GEM models in general are complex and have limitations in the immune environment and animal cost. Their application in the context of MM heterogeneity will require the use of multiple cell lines according to their molecular profile. To achieve sound results with significant statistical value, including kinetics and dose-effect relationships, a large number of animals would also be needed, unless solid ancillary results are available. Besides economic issues, the 3R rules (Replacement, Reduction, and Refinemen) on the use of animals in scientific procedures are recommended. The identification of biomarkers to follow tumor evolution in response to anti-cancer drugs is of importance to limit the number of animals (117). Lower attrition rates for oncology drugs would be obtained with more predictive models (72, 104, 117, 118). Consequently, it is necessary to develop alternatives for replacement, robust and reproducible bases for reduction, and the use of advanced technologies for refinement (119).

Appropriately designed and analyzed preclinical assays are required (72), with the aim to identify new anti-cancer compounds for MM and novel biomarkers for sensitivity or resistance, which are essential to predict the tumor response. Although animal models are considered to be the most relevant, the development of sophisticated *in vitro* multicellular models should be encouraged. The continuing increase in the knowledge about mesothelial carcinogenesis will permit the use of more pertinent cell models that represent the MM tumor. New approaches not yet used in MM should be explored, including organ-on-a-chip technologies or *in silico* biological systems using computational modeling and machine learning (120, 121). Powerful technological tools should allow researchers to establish models with MM cells that grow in a more accurate tumor microenvironment, and possible *in situ* molecular analyses of tumor cells. The use of well-characterized tumor cells, classified in subgroups of molecular classifications or characterized by histo-molecular gradients, is particularly important regarding the molecular heterogeneity of human MM. This endeavor should allow researchers to obtain representative results of a given type of tumors.

The ongoing preclinical models should be improved with regard to precision, reproducibility, and predictivity, and the results should be supported by different approaches. Some standardization might be helpful. The use of existing consortia and/or the development of new consortia will allow the inclusion of more tumor samples in studies and increase the number of relevant cell models. These factors will enable researchers to adequately cover mesothelioma heterogeneity and be able to afford the high costs of new technologies. Some authors have recommended improving the reliability of preclinical cancer studies by using detailed information on the experimental methodology, different approaches, the publication of negative data, and better dialogue between physicians and scientists (122, 123). These factors are particularly important within the actual context of precision medicine, which implements complex methodologies and multidisciplinary investigations and has a high cost.

## AUTHOR CONTRIBUTIONS

M-CJ and CB prepared the figures. CB, M-CJ, and DJ wrote the review.

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