Preclinical Models of Malignant Mesothelioma

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INTRODUCTION

Malignant mesothelioma (MM) is a treatment-resistant malignancy causally linked to asbestos exposure. Despite recent advances in therapeutic modalities, MM patients usually die within 1 year following diagnosis. MM is particularly lethal in patients with pleural disease, particularly those whose tumors have sarcomatoid features (1). Consequently, in vivo models of MM are needed to investigate MM disease pathogenesis and to provide accurate preclinical models for identifying new therapies that might move forward in clinical trials.

We here summarize where we stand with regard to existing models of MM and how they might be further improved. All the desirable features will be unlikely found in a single model, but the disease evolving in the model should mimic at least several of the salient features of human MM, such as its pathology, its gene expression patterns, the genetic driver lesions, and the inflammatory phenotype that is characteristic for MM. In view of the inflammatory phenotype of MM and the prominent role the immune system fulfills in either promoting or impairing tumor development, models exhibiting this specific feature should also be part of the armamentarium. Preferentially, the model should also exhibit a reproducible and short latency period as to permit intervention studies. The models—mostly encompassing small rodents—range from graft models in which human MM cell lines or patient-derived tumor fragments are implanted to complex conditional tumor suppressor gene knockout/oncogene mouse models.
SOMATIC GENETIC AND SIGNALING ALTERATIONS IN HUMAN MESOTHELIOMA

There is abundant evidence that inactivating somatic mutations and deletions of the tumor suppressor genes (TSGs) BAP1, CDKN2A, and NF2 represent the most frequent genetic lesions in human malignant pleural mesothelioma (MPM) (2–13). Moreover, losses of these three TSGs are frequently seen in various combinations in a given MPM (7, 14). The notion that loss of these particular TSGs is so predominant implies that MPM development critically depends on the cellular signaling pathways that are guarded by these genes.

CDKN2A encodes p16INK4a and p14ARF, two tumor suppressors that, respectively, regulate the Rb and p53 cell cycle pathways. p14ARF is a component of the p53 pathway, and TP53 alterations have also been observed in some MPMs (6, 15). In fact, a recent report that compared next-generation sequencing of two series of MPMs—one from The Cancer Genome Atlas (TCGA) (13) and the second from a Harvard series (12)—revealed only four “significantly mutated genes at a false discovery rate of <0.05” common to the two studies: BAP1, NF2, TP53, and SETD2, each of which showed prominent levels of inactivating nonsense, frameshift, and splice-site mutations, consistent with their putative roles as driver loss-of-function lesions in this malignancy (13). In the TCGA data set, focal deletions were found to affect several TSGs, especially CDKN2A, with deep, apparently homozygous deletions occurring in 36/73 (49%) tumors and single-copy losses in 5 others (7%) (13). In the Harvard series, Bueno et al. found copy number losses of CDKN2A in 48/95 (51%) MPMs (12). In a deletion mapping analysis, homozygous CDKN2A deletions were identified in 36 of 40 (90%) human MPM cell lines tested, while homozygous deletions of the adjacent locus CDKN2B occurred in most—i.e., 32/36—of these same cell lines (6). Experiments in mice have shown that the Cdkn2b also exhibits a tumor suppressor role in MPM, as its deletion concomitant with Cdkn2a further accelerates MPM development (our unpublished results) offering a rationale for the predominant deletion of all three tumor suppressors in this locus in MPM.

Unlike these specific TSGs, mutations of protooncogenes are seldom identified in MPM. Moreover, in the TCGA cohort, no activating mutations were observed in genes encoding components of the MAPK or PI3K/AKT pathways (13). However, both PI3K/AKT/mTOR and RAS/MAPK pathways were upregulated in this series, and they were each associated with a poor-prognosis. Moreover, despite a rarity of mutations of PTEN in MPM, earlier immunohistochemical (IHC) studies revealed diminished PTEN protein expression in 16 to 62% of MMs in several studies (16–18). Additionally, various receptor tyrosine kinases (RTKs) were shown to be frequently overexpressed and/or activated in MPM, resulting in activation of proliferation and pro-survival signals through the PI3K/AKT/mTOR signaling pathway (7, 19–21). Thus, it is not surprising that phospho-AKT immunostaining is observed in a high percentage (65–84%) of human MPMs (6, 16, 22, 23).

In view of the prominence of TSG inactivation and the relatively rare oncogenic gain-of-function mutations in MM, high-throughput chemical inhibitor screens and gene expression analyses have been performed in MM cell lines to identify unique vulnerabilities. Chemical screens pointed to increased sensitivity to FGFR inhibitors in a subset of the MPM cell lines. This corresponded with higher FGFR3 expression specifically in cell lines not expressing BAP1 (24). BAP1-deficient MM also showed augmented sensitivity to TRAIL (25). Furthermore, loss of BAP1 function was found associated with increased expression of EZH2, with concomitant widespread epigenetic gene silencing sensitizing the cells to EZH2 inhibitors (26), whereas the impaired argininosuccinate synthase 1 (ASS1) expression likely as a result of enhanced EZH2 levels sensitized cells to arginine deprivation (27, 28). In addition, BAP1-depleted cells showed increased sensitivity to PARP inhibition (29). Another vulnerability relates to the co-deletion of CDKN2A and the nearby methylthioadenosine phosphorylase (MTAP) gene (30), the latter rendering cells dependent on protein arginine methyltransferase (PRMT5) (31, 32). NF2 depletion leads to dysregulation of the Hippo pathway by activating the transcriptional co-activator YAP1 and its association with the TEAD family of transcription factors, resulting in up-regulation of genes that promote cell proliferation and inhibit cell death. Inhibitors that disrupt the YAP/TAZ-TEAD complex are not yet available but could serve as promising drugs in view of the strong dependence of MM on activation of the Hippo pathway (33). MM also shows overexpression of RTKs such as MET and downstream PI3K, making inhibitors targeting components of this pathway other promising therapies for this disease (21). Therefore, there are a number of potential vulnerabilities that are worth exploring both as single agents and as combinations in the various preclinical models of MM.

RODENTS AS MODELS OF ASBESTOS CARCINOGENICITY AND MESOTHELIOMA PATHOGENESIS

Numerous investigators have induced MM in rats and mice via injection or inhalation of asbestos fibers (34) or in hamsters through exposure to SV40 (35). Notably, several studies have shown that the MMMs induced in rats via asbestos inhalation do not exhibit cytogenetic or gene expression patterns similar to those seen in their human tumor counterparts nor do they show inactivation of genes implicated as drivers in human MM (36–39). Studies in the laboratory rat, beginning in the 1960’s, documented that various forms of asbestos and other mineral fibers inoculated intrapleurally/intrathoracically (IT) developed MPM (40). Erionite, the zeolite mineral fiber that is linked to the MM epidemic in near Cappadocia, Turkey (41, 42) was shown to be more carcinogenic than asbestos in IT injection or inhalation studies (43).

While the rat was favored over the mouse as a model for mineral fiber studies due to its larger pleural space for inoculation and “…its more suitable nasal passage architecture
for inhalation studies, some of the early investigations did use mice for IT inoculation of amphiboles and serpentine mineral fibers; however, fibrosis and granulomas were mainly observed (44), with occasional papillary carcinomas seen in inhalation experiments (45). Subsequent carcinogenicity studies using intraperitoneally (i.p.)-inoculated asbestos or zeolite fibers resulted in MMs in more than 20% of wild type mice (46). Over the last two decades, various laboratories have reported variable MM incidences and survival rates in wild type mice that have been injected i.p. with asbestos fibers (6, 38, 47–56), due at least in part to the use of differing types, dimensions and amounts of fibers used, whether the injections were given chronically or as a bolus injection, the length of time the animals were followed, and variations in the genetic background of the mice.

Genetically engineered mouse (GEM) models, typically harboring heterozygous whole-body germline mutations, have been used to assess whether loss of TSGs implicated in human MPM accelerate tumor formation. Different groups have performed such experiments with GEM models carrying mutations of MM-related genes. An early investigation used Tp53-deficient mice (38, 47), with mice injected i.p. with crocidolite weekly for 22 weeks. Tp53+/− mice developed a high incidence (76%) of MMs (median latency, 44 weeks) vs. a32% incidence (76%) of MMs (median latency, 44 weeks) vs. a32% heterozygous deletions of p14ARF (19Arf in the mouse). To test the relative contribution of both gene products results in further cooperation to accelerate BAP1 mutations in asbestos-induced MM development and progression.

The data demonstrate co-carcinogenicity between asbestos and SV40. The use of Bap1 knockout models has shown that heterozygosity in the germline predisposes to asbestos-induced MM (53, 59), and similar results were obtained with two knock-in models (54) that harbored different germline mutations that were identical to the ones found in two BAP1 tumor predisposition syndrome (BAP1-TPDS) families that exhibited a very high incidence of MM (8). MM cells from Bap1+/− mice showed biallelic inactivation of Bap1 (53). Collectively, these data indicate that human BAP1 mutation carriers have are more prone to the carcinogenic effects of asbestos, even when exposed to small amounts of these fibers (59); when compared to the general population.

Other work has recently demonstrated cooperation between Nf2 and Cdkn2a in MM development in asbestos-exposed Nf2+/−;Cdkn2a+/− mice, which exhibited significantly hastened tumor onset and disease progression vs. similarly exposed Nf2+/− and wild-type cohorts (56). These studies also showed that tumors from Nf2+/−;Cdkn2a+/− mice had enhanced metastatic potential and an increased cancer stem cell population, in connection with p53/miR-34a-dependent activation of c-Met.

Since chronic inflammation may contribute to the formation of many types of malignancy, including MM, some investigators have employed mouse models for studies of asbestos-mediated inflammation. In one such study, Nf2+/−;Cdkn2a+/− mice were used to test if inflammation-related IL-1β release promotes MM formation (55). Exposure of Nf2+/−;Cdkn2a+/− mice to asbestos in the presence of an IL-1 receptor (IL-1R) antagonist known as anakinra resulted in a significant delay MM development compared to that of asbestos-exposed mice given a vehicle control, i.e., 33 vs. ~22.5 weeks, respectively (55). Overall, this work suggested that inflammation-related IL-1β/IL-1R signaling is linked to the formation of asbestos-induced MM. Moreover, the data demonstrate the usefulness of this model for gene-environment and/or “chemoprevention” studies.

Another mouse model, MexTAg, has been used to demonstrate co-carcinogenicity between asbestos and SV40. The investigators used the mesothelin gene promoter to express SV40 large T antigen specifically in the mesothelial lining (49, 60). Several MexTAg mouse lines were created with varying copies (1–100) of the oncogenic transgene. The animals generally do not develop spontaneous MM. However, after i.p. injection of asbestos, 100% of the MexTAg mice developed MM, with disease onset occurring after 20–40 weeks vs. after 50–100 weeks in the ~25% of wild type mice that developed MM. The investigators concluded that MexTAg mice are well-suited not only basic research, but also for testing the potential of dietary or pharmacological chemoprevention studies of MM (49). To illustrate the utility of MexTAg mice for preclinical studies,
Robinson et al. tested the effects of gemcitabine, a cytotoxic drug that has been shown to have some efficacy in the human disease (60). MexTAg mice treated with vehicle had a median survival of 33 vs. 48 weeks in the gemcitabine-treated cohort. In another investigation with MexTAg mice, treatment with celecoxib, a COX-2 inhibitor, did not diminish the rate of asbestos-induced MM, despite the fact that COX-2 is frequently overexpressed in human MM and correlates with poor prognosis (60). While the MexTAg model has several advantages (100% MM penetrance, short median survival), it does not have any of the genetic hallmarks attributed to the human disease, and a causative association between SV40 and human MM is now disproven (61, 62). However, in one study, gene expression profiling of MMs from MexTAg mice “…had a concordant set of deregulated genes compared to normal mesothelial cells that overlapped with the deregulated genes between human MMs and mesothelial cells” (63).

**CONDITIONAL MOUSE MODELS OF MESOTHELIOMA AS PRECLINICAL TOOLS**

Since specific genetic driver lesions had been repeatedly found to be associated with human MM by the year 2008, particularly alterations of the CDKN2A, NF2, and TP53, Jongsma et al. decided to establish whether various genetic alterations affecting the same signaling pathways that are dysregulated in the human disease counterpart might similarly induce MM in rodents in the absence of carcinogenic exposure to asbestos (64). Thus, they generated a variety of mutant mice carrying deletions in the NF2/merlin, p53, and/or Ink4a pathways, hypothesizing that mice with one or more of these combinations might represent an appropriate model of human MM. To avoid possible issues such as embryonic lethality due to germline homozygous deletion of one or more targeted genes, mice with conditional knockout (CKO) of various TSGs were used in combination with the Cre-loxP system (65). Locomotorial inactivation of the TSG(s) was carried out by injecting adenoviruses expressing the Cre recombinase (65). Upon injecting adeno-Cre into the pleural space of Rosa26 LacZ reporter mice, the investigators demonstrated expression of β-galactosidase specifically in the mesothelium (64). Moreover, MPMs arose in both Nf2/Tp53 and Nf2;p16Ink4a/p19Arf CKO mice at a high frequency and short latency (20 and 30 weeks, respectively) following IT inoculation of adeno-Cre, and the tumors closely mimicked the phenotype of human MPM. Thus, these mice hold promise as a rapid, non-carcinogenic model system for preclinical selection of new combination therapies and for testing novel targeted agents.

BAP1-TPDS patients with MM have a significantly better long-term survival compared to sporadic MM patients, i.e., those without a heritable variant (11, 66). However, it remained unclear whether somatic mutations/deletions of BAP1 have a similarly favorable prognosis in sporadic MM, or if somatic BAP1 alterations are a poor prognostic marker, as is the case for uveal melanoma and clear cell renal cell carcinoma (67, 68). Furthermore, although most human MMs exhibit somatic alterations of BAP1, NF2, and/or CDKN2A—with 25/74 cases of MPM in the TCGA series having alterations of all three TSGs in combination (13)—it was not known if loss of BAP1 could cooperate with the inactivation of NF2 and/or CDKN2A to initiate a more aggressive form of MM. To address this possibility experimentally, Kukuyan et al. used CKO models, including a Bap1flo/flo mouse they generated (69). Various combinations of deletions of Bap1, Cdkn2a, and Nf2 were introduced in the pleural cavity of the mice, focusing on the contribution of Bap1 loss. While homozygous CKO of any one of these TSGs alone gave rise to few or no MMs—similar to the results of Jongsma et al. (64)—deletion of Bap1 cooperated with deletion of either Nf2 or Cdkn2a to promote MM formation in about 20% of double-CKO mice. In contrast, a much higher incidence (22/26, 85%) of MMs was observed in Bap1+/−;Nf2+/−;Cdkn2a+/− mice injected IT with adeno-Cre (triple-CKO mice). Onset of MM was rapid in the triple-CKO mice (median survival, 12 weeks), and tumors from these mice were consistently high-grade and invasive. With regard to histological subtype, notably no epithelioid MMs were observed with any of the mouse genotypes. Sarcomatoid MMs predominated, with the only exception being the Bap1;Nf2 double-CKO cohort, in which 6 of 7 MMs showed mixed (biphasic) histology. The MMs observed in triple-CKO mice showed enrichment for genes that are transcriptionally controlled by the polycomb repressive complex 2 (PRC2) (69). The findings suggested that loss of Bap1 contributes to MM progression, at least partially, via loss of PRC2-mediated repression of oncogenic target genes that were identified, suggesting a novel avenue for therapeutic intervention (69).

To explore the role of individual components of the Cdkn2a locus by comparing models in which Cdkn2a (including p19Arf) were disrupted with or without concomitant loss of Cdkn2b Badhai et al. showed that the additional disruption of Cdkn2b further added to the aggressiveness of the resulting MMs, providing also an explanation for the predominance of deletion of the complete CDKN2A-CDKN2B locus in human MM over point mutations in CDKN2A (Badhai et al., submitted). Because CDKN2A deletions encompassing the sequence encoding p14ARF, a component of the p53 pathway, have been documented in 90% of human MM cell lines (6) and TP53 is altered in about 15% of primary MMs, and because the PI3K/PTEN/AKT pathway is activated in most human MPMs, Sementino et al. decided to determine if alterations affecting the same pathways would also induce MM in mice (70). This was thought worthwhile, given that p53 helps mediate the DNA damage response and that AKT regulates neoplastic cell survival and therapeutic resistance. The investigators demonstrated that while neither adeno-Cre-mediated homozygous deletion of Tp53 or Pten alone in the mesothelium was sufficient to induce MM formation, compound deletion of these two TSGs resulted in rapid, aggressive peritoneal and pleural MMs (median latency: 9 and 19 weeks, respectively). A longer term follow-up study of the Tp53−/− cohort revealed MMs in 0/12 mice injected with adeno-Cre i.p. and 0/10 mice injected IT; among the Pten−/− cohort, MMs were observed in 0/12 mice injected i.p. and 1/10 injected IT (Sementino et al., unpublished data). In the Pten−/−;Tp53−/− cohort, 23/25 (92%) mice injected i.p. developed MM, whereas
19/34 (56%) mice injected IT showed MM, with 14 histiocytic sarcomas also seen in this group.

Given the high penetrance and rapid development of MMs in *Ptet*\(^{+/+}\);*Tp53*\(^{+/+}\) mice inoculated i.p., and the frequent involvement of p14ARF/p53 and PI3K/PTEN/AKT pathways in human MM, this GEM model holds promise for preclinical work. However, this model does have certain limitations, such as for testing agents designed to reactivate the normal cellular functions of Pten and Tp53. For instance, given that this model has homozygous loss of *Tp53*, this precludes studies of a drug such as RITA, which reactivates p53's pro-apoptotic function in tumor cells that preserve expression of mutant or wild-type p53 (71). To elude this issue using an agent such as RITA, this mouse model might be modified such that only a single *Tp53* allele were deleted, i.e., by using *Ptet*\(^{+/+}\);*Tp53*\(^{+/-}\) mice. A second shortcoming with regard to the translational relevance of the *Ptet*\(^{+/+}\);*Tp53*\(^{+/+}\) model is that somatic mutations of other TSGs considered to be hallmarks in human MM progression usually do not occur in tumors from these animals. However, the fact that the MMs in this model repeatedly show sarcomatoid or biphasic histology with very short latency, especially in mice injected i.p., provides advantages for certain preclinical applications.

### GRAFT MODELS OF MESOTHELIOMA

Many human MM cell lines have been established over the years and used in numerous *in vitro* studies. They are also exploited for *in vivo* experiments, usually for testing their tumorigenicity and the efficacy of small molecule inhibitors as a prelude for evaluating these compounds in clinical trials. Due to often long-term *in vitro* propagation, these cell lines have invariably acquired (epi)genetic alterations that facilitate their propagation in cell culture, resulting in new vulnerabilities and resistance features. This is one of the reasons why treatments that are effective in these graft models often do not well-translate to human. Furthermore, the requirement to use immunodeficient mice as a host for these graft experiments complicates assessment of immunomodulating effects. Patient-derived xenograft (PDX) models, in which tumor fragments are grafted directly into immunodeficient recipient hosts, more closely resemble the human condition and usually retain their human stromal components for a number of passages. The capacity to establish PDX lines also correlates with the aggressiveness of the tumor in man (72). Studies in PDX models permit addressing specific questions that are difficult to assess in solely mouse based models such as inter- and intra-tumor heterogeneity as well as features imposed by the distinct genetic backgrounds (73). As potential drawbacks, we note that propagation has to be performed in immunodeficient backgrounds and retrofitting these models with a functional human immune system from the patient from which the tumor was obtained (humanized models) is still in an early stage of development (74) and also practically very demanding.

Experienced investigators seeking a mouse model that may faithfully reflect human MPM pathobiology may also find an orthotopic, intrapleural model such as the one described by Servais et al. (75) useful for preclinical therapeutic studies. This tumor model recapitulates human pleural anatomy/microenvironment and can be used in combination with quantitative, non-invasive imaging for bioluminescent monitoring of tumor burden. The parietal pleural surface contains lymphatics that offer escape of MM cells into the systemic circulation, and this immunocompetent orthotopic model of pleural cancer permit studies of inflammation on tumor progression as well (75). However, as noted by the authors, for studies of therapies targeting human antigens, immunodeficient models are required in order to perform studies on xenografted human cancer cell lines.

### ARE WE MISSING ANYTHING?

First of all, it is worth emphasizing that the choice of the model depends on the question asked. Furthermore, a mouse is not a “small human” and we need to accept that we cannot simply extrapolate findings from such a model to the human condition. However, models can teach us important biological principles and can provide us with therapeutic concepts worth testing in clinical settings notwithstanding the evolutionary distance between man and mouse. Where possible, we should try to align the model on the basis of molecular aberrations found in humans, e.g., by introducing similar driver lesions in the right target cell and using comparable external carcinogens if applicable, e.g., asbestos. Evidently, PDX models might be very valuable to assess intrinsic tumor heterogeneity and to evaluate their response to drug combinations. For immunotherapy studies, it will be important to use a model with a functional immune system. To permit effective immunotherapy studies in MM mouse models, it will be important to establish these in a defined genetic background (e.g., BL6, the “work horse” of immunologists) in order to permit isogenic graft studies. Fortunately, current Crispr/Cas9 engineering has made the generation of complex conditional MM models relatively easy. This should facilitate the testing of new promising intervention strategies for this highly lethal cancer.

### AUTHOR CONTRIBUTIONS

JT and AB wrote the paper and are accountable for the content of the work.

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Conflict of Interest: JT has a patent on BAP1 mutation testing and has provided legal consultation regarding the role of germline mutations of BAP1 in mesothelioma.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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