Review Article

# *In vitro* experimental models of mesothelioma revisited

**Anand Singh, Nathanael Pruett, Chuong D. Hoang**

Section of Thoracic Surgery, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

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*Correspondence to:* Chuong D. Hoang, MD. Thoracic and Gastrointestinal Oncology Branch, Section of Thoracic Surgery, National Cancer Institute, National Institutes of Health, CCR and The Clinical Center, 10 Center Drive, Room 4-3940, Mail code 1201, Bethesda, MD 20892, USA.
Email: chuong.hoang@nih.gov.

**Abstract:** Malignant pleural mesothelioma (MPM) is a biologically unusual, highly aggressive cancer that defies current multimodality treatments. Epidemiologic data suggest that this malignancy has not abated despite increasingly strict environmental regulations on asbestos, the putative causative agent for sporadic cases. An incomplete understanding of all the factors mechanistically driving mesothelioma is largely responsible for the current lack of curative treatments. Many approaches have been employed to ascertain the step-by-step molecular events involved in mesothelioma oncogenesis including *in vitro*, small animal *in vivo*, and human experimental models; though clearly defined, druggable mechanisms still are elusive. Importantly, the foundation of the latest accepted model of tumor initiation is derived from *in vitro* systems. A thorough review of *in vitro* mesothelioma oncogenesis models may suggest further opportunities for discovery.

**Keywords:** Mesothelioma; transformation; oncogenesis; *in vitro*; asbestos

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## Introduction

Malignant pleural mesothelioma (MPM) is the most common type of mesothelioma and remains an incurable malignancy with few treatment options. Since 2003 when cisplatin and pemetrexed combination therapy was validated as the standard drug regimen for clinical treatment (1), there has been minimal improvement in long-term survival. Incremental progress has been achieved in eligible MPM patients with the recent addition of bevacizumab (2) to the standard two-drug paradigm, but even this newer combination regimen lacks a strong specific biologic rationale against MPM. Perhaps a systematic reappraisal of the current oncogenesis models for MPM is warranted to refocus research efforts aimed at identifying critical molecular pathways. For the purpose of this review, specific attention is given to analysis of *in vitro* transformation models of MPM that have provided insights into the various molecular mechanisms and genetic alterations at the core of the MPM malignant state. With a critical understanding of the pros and cons of each *in vitro* model scheme, principles can be derived to guide future and ongoing research towards a consistent and more physiologically accurate explanation of MPM oncogenesis.

## Clinical trends

MPM is an aggressive cancer that arises from the mesothelial lining of the pleura, peritoneum and pericardium, and rarely from the tunica vaginalis of the testis (3). Approximately 80% of mesothelioma cases are pleural in origin and are defined as MPM (4). MPM is highly associated with occupational exposure to asbestos fibers which are widely accepted as the primary causative agent (5). Although asbestos has been nationally banned in many developed regions of the world including Europe,

Scandinavia, United Kingdom, Japan, and Australia current notable exceptions exist such as China, Russia, India, Brazil, Canada and the United States (6). Of further note, Canada has implemented legislation to ban asbestos use by 2018 (7). In the United States significant asbestos abatement via regulatory actions of the Environmental Protection Agency have served to limit asbestos exposure as much as practical to the general public (8).

Subsequently, the incidence and prevalence of MPM continue to show alarming trends worldwide. In the United States, for example, even though predictions suggested that the incidence of MPM should have peaked in the early 2000s (9), the incidence rate per 100,000 people, shows no change since 1975 (10). Currently, the annual incidence of MPM in the United States remains approximately 3,200 individuals affected (11). Further, the latest analysis from the Centers for Disease Control and Prevention observed that the annual number of deaths from mesothelioma continued to unexpectedly increase by 4.8% (P for linear time trend <0.001) overall to 2,579 cases in 2015. And the underlying main factor for this rise was increased deaths among persons aged ≥85 years (8). In other countries, current predictions of MPM incidence have not yet reached peak levels. By 2020–2030 and beyond, in industrialized nations alone, the increase is likely to affect thousands of people (12). For the foreseeable future, the prevalence of MPM remains a significant cancer type and the incidence continues to increase worldwide, making mesothelioma a major international health problem (12,13).

## Pathogenesis

MPM is a highly complex tumor at the genetic level lacking consistent molecular patterns that would inform on obvious treatment approaches, but which suggest there are multiple active oncogenic programs cooperating to drive this malignancy (14,15). To date, much of the new therapeutic approaches that have been tested in clinical trials have focused intervention efforts on one biological pathway or molecule that is deemed critical to the development and ongoing growth of MPM. Therefore, part of the reason why modern treatments fail to yield a durable cure is because the biological basis of the disease is not fully understood.

In the latest summaries (5,14,16), a current accepted view of MPM pathogenesis derives from *in vitro* studies of human and murine cells, *in vivo* murine models, as well as indirect evidence from human surgical or autopsy studies. In brief, MPM oncogenesis is hypothesized to consist of the following major steps: (I) a fraction of inhaled asbestos fibers preferentially reach distal lung alveoli based on particle shape and size characteristics; (II) asbestos fibers transit to the pleural space where drainage mechanisms of stomata (μm in size) emptying into a lymphatic network act to clear the fibers out of the chest; (III) the fibers, because of their shape and size, get retained at stomata on the parietal pleural surface; (IV) macrophages migrate to the pleural space and attempt to phagocytose these fibers, but since they cannot be internalized completely, frustrated phagocytosis ensues to form "black spots" initiating a pro-inflammatory cascade of chemical mediators (reactive oxygen species, cytokines, and growth factors) and likely multiple signaling pathways cooperate to promote oncogenesis; (V) in parallel, there is direct interaction of asbestos fibers with human mesothelial cells (HMC) which incur genotoxic effects and undergo damage necrosis, further perpetuating a cascade of chronic inflammation primarily at the parietal pleura; (VI) presumably, in some parietal HMC, there is enough inherent or acquired resistance to asbestos effects (apoptosis/cell death) that is mediated by nuclear factor-kappa B (NF-κB) signaling, to foster their survival and proliferation (17,18); (VII) in the setting of ongoing asbestos-induced deoxyribonucleic acid (DNA) damage, genetic abnormalities accumulate in those surviving HMC; and finally (VIII) asbestos-induced genetic instability in parietal HMC culminate in a multi-step process of cancer evolution.

A highly controversial topic of MPM pathogenesis concerns the role of simian virus 40 (SV40). While SV40 large-T antigen (Tag) directly interacts with transformation-related protein 53 gene (p53) and retinoblastoma (Rb) tumor suppressor pathways in MPM cells (19), and has been used as a co-factor with asbestos fibers in numerous *in vitro* (reviewed here in our article) and small animal studies of mesothelial cellular transformation, its direct relevance in human MPM specimens has been strongly questioned. Many human epidemiologic studies performed to date do not support any clinically relevant association between SV40 and human MPM (20). Multiple research efforts (small and multi-institutional groups) have been unable to convincingly detect SV40 genetic sequences in human MPM (21-25). Perhaps more notably, ongoing updates of MPM now have either omitted any further discussion of SV40 as a causative agent (14,16) or suggest that this viral contamination theory should be discarded (5).

 

## Current knowledge gaps

Critical review of the data supporting the current oncogenesis framework exposes persisting assumptions in our knowledge that contribute to unsolved mechanistic links. A few areas of knowledge gaps regarding MPM pathobiology are highlighted:

(I) It remains unknown how airborne asbestos fibers traverse the lung interstitium to preferentially affect the pleura and incite MPM as opposed to primarily causing disease in lung parenchyma. The lymphatic network draining into stomata on parietal pleura has only been observed in small animal models, never conclusively demonstrated in humans (16,26). Additionally, there is virtually no knowledge about the kinetics of asbestos fiber translocation and deposition in human pleura (27);

(II) While the majority of studies assume the cell of origin for MPM is the pleural mesothelial cell, a small body of literature theorizes other cell types could be responsible such as mesothelial progenitor cells that influx to parietal pleura or are induced in parietal regions of cellular damage caused by asbestos (5,28);

(III) Related to this concept is the controversial field of cancer stem cell biology applied to solid tumors for which there is a paucity of literature to support this notion in MPM (29-32). These reports are hampered by lack of a consistent and specific stem cell marker(s), and lack of reproducibility from serial dilution experiments of primary MPM samples;

(IV) Although it is recognized that inflammation plays a role in MPM pathogenesis, the direct molecular mechanism(s), if any, linking inflammation to cancer development have not been described in detail. It is well-accepted that NF-κB generally mediates survival from asbestos-induced effects (cell killing) in some, not all HMC; and it is those surviving HMC which go on to form a tumor (17,18). The mechanism(s) that influence selection of HMC sub-population(s) that can survive the initial killing induced by asbestos remains unknown;

(V) The early molecular events through which asbestos causes mesothelial cell transformation have yet to be fully understood. Possible mechanisms have been proposed for the pathogenesis of MPM that describe effects on asbestos-exposed HMC and macrophages as: (i) generation of reactive oxygen species, leading to DNA damage and chromosomal alterations that are the basis for development of malignant cells (33); (ii) activation of multiple receptor tyrosine kinases with constitutive proliferation, for example, via epidermal growth factor receptor etc. (34); and (iii) secretion of high mobility group protein B1 (HMGB1) from damaged HMC (although it is not well explained why this is specific to HMC undergoing damage necrosis nor why this is responsible for major effects as multiple other damage-associated molecules are released) to perpetuate a chronic auto-inflammatory cascade via toll-like receptors and receptor for advanced glycation end products (35). But little is understood in terms of which mechanism is activated preferentially by asbestos exposure, nor which of these mechanisms have a primary role during any time points of cellular transformation;

(VI) Also, it cannot be explained in the current framework how MPM progresses to cover an enormous surface area spreading out laterally. Why does MPM not grow and enlarge as a spheroid volume similar to all other solid tumors? A possible hypothesis to partly explain this characteristic would require multiple metachronous foci of mesothelial cells acquiring malignant growth, but direct evidence to explain this phenomenon is unavailable;

(VII) Recent data indicates a polyclonal cell origin for MPM (36). This observation could explain clinical experience that recognizes MPM behaving as a composite of multiple tumors with inter- and intratumor genetic heterogeneity (14,16), contributing to the ability of MPM to generally resist all modes of therapy. But overall, the impact of this finding on MPM biology requires more investigation;

(VIII) There are few consistent and highly recurrent genetic mutations acknowledged as oncogenic drivers despite a multitude of chromosomal and genetic MPM profiling studies (14,37). In terms of somatic alterations, the overall mutational burden of MPM is low among various solid tumors most comparable to neuroblastomas (38,39), and, to date, only three driver genes

 

[cyclin-dependent kinase inhibitor 2A gene (CDKN2A), neurofibromin 2 gene (NF2), and BRCA1 associated protein-1 gene (BAP1)] are commonly recognized (40).

Remarkably, the notion of chronic inflammation initiated by asbestos remains a hypothesis without direct human (*in vivo*) mechanisms delineated (16). Reliable inhalation studies with well-characterized aerosols of various asbestos fibers in long-term exposure animal models have not been reported due to cost, complexity, and lack of specialized instrumentation. Thus, few, if any, *in vivo* human mechanistic studies confirm the widely held concept of chronic inflammation associated with asbestos (27). Largely, it is indirect evidence that supports the pervasive role of inflammation in MPM pathogenesis. Elevated interleukin (IL)-6 levels in serum and pleural effusions of patients with MPM have been observed to generally support involvement of an inflammatory condition associated with malignancy in the context of asbestos (41,42). Also, pathologic analyses of MPM generally support the notion of intense inflammatory infiltrating cells of the tumor microenvironment in resected specimens (43).

## Basis of knowledge gaps

Multiple factors collude to obscure the precise pathogenetic mechanisms specific to MPM. Likely what is required for a comprehensive understanding of the pathologic processes in MPM (or any cancer) is a synthesis of *in vitro*, *in vivo* (animal), and human modeling data sets. While current technology does not yet facilitate such a seamless integration of modeling systems, much complementary information can still be curated from each. Tumor tissue specimens represent a diverse and complex mixture of malignant cells and other cell types that, en masse, have achieved multiple milestones characteristic of cancer. Despite increasingly sophisticated technologies to assess genetic alterations of the whole tumor mass, the results may reflect relatively late, advanced cancer mass changes not easily treated by systemic chemotherapeutics or molecular targeted agents. The next alternative to better assess and dissect cancer processes is to use animal models that can reveal the complexity of tumorigenic processes in a living system, however visualization of molecular or biologic events step-by-step and quantitation of data is typically a prohibitive technical challenge. Additionally, extrapolating animal model responses to human cancer patients has yet to fully realize direct translational successes (44). A complementary and still useful type of system is the classical *in vitro* model of cultured cell lines which permit direct manipulation and precise dissection of gene regulatory networks and predominant signaling pathways (45). In fact, the selection of an appropriate and physiologically relevant *in vitro* model is important for the investigation of chromosomal changes, epigenetics, initiation and progression, and deregulation of apoptosis and proliferation, etc. However, *in vitro* systems have many limitations including: lack of cellular heterogeneity/complexity similar to the original tumor, or genotypic and phenotypic drifting away from the original tumor after prolonged culture time, etc. (46). Perhaps a review of MPM *in vitro* models could suggest further experimental designs that better address some of the knowledge gaps in MPM pathobiology as mentioned here.

## *In vitro* models of oncogenesis

Since the mechanism(s) underlying development of MPM continue to be elucidated, there is no consensus on an ideal *in vitro* model. Many groups have sought to propose *in vitro* models of MPM development, although each has pros and cons in their design and execution (*Table 1*).

### *Transformation by gene transfer*

The first *in vitro* model of MPM oncogenesis as reported by Reddel *et al.* was created by transfecting guanosine-5'-triphosphate-bound isoform of p21 ras (constitutively activated) gene (EJ-ras) oncogene into an immortalized HMC line MeT-5A (47). The EJ-ras transfected cells were tumorigenic, able to form tumors in nude mice, while untransfected MeT-5A cells did not form tumors. Prior to this work, it was known that primary HMC did not similarly transform with EJ-ras transfection (58), so these authors deduced that multiple, instead of single, molecular steps are likely required by HMC before producing a malignant phenotype. The MeT-5A cells, derived from pleural mesothelium, were immortalized by insertion of Tag (59) which inhibits both p53 and Rb pathways likely providing a permissive genetic background for full cellular transformation to a malignant phenotype. Implicit in this model is the assumption of HMC being the cell of origin for MPM. Along this same conceptual framework of constitutive oncogene(s) being responsible for HMC transformation and oncogenesis, Van der Meeren *et al.* transfected MeT-5A cells with platelet-derived growth factor-A gene which formed tumors in nude mice allowing

 

**Table 1** Human mesothelial cell (HMC) transformation models

| Model system | Condition(s) | Transform (t, weeks) | Validation | Summary | Refs. |
|---|---|---|---|---|---|
| MeT-5A | EJ-ras overexpression | 6–12 | Tumor xenografts | EJ-ras is an oncogene in MPM | Reddel, 1989 (47) |
| | PDGF-A overexpression | 11–12 | Tumor xenografts | PDGF-A is an oncogene in MPM | Van der Meeren, 1993 (48) |
| HMC (pleural fluid) | SV40 ± crocidolite | 6–8 | Colony foci, soft agar | Co-carcinogens SV40, asbestos, intact p53 | Bocchetta, 2000 (49) |
| MeT-5A | TNFα ± IL-1β ± erionite | >16 | Colony foci, soft agar | Multiple cytokines can transform, but not erionite alone | Wang, 2004 (50) |
| HMC (pleural fluid) | SV40 + amosite | 4–8 | Monolayer foci | PI3K/Akt | Cacciotti, 2005 (51) |
| | Erionite | 9 | Monolayer foci | Akt, NFKB, ERK1/2 | Bertino, 2007 (52) |
| | Macrophage + erionite | 8 | 3D foci | HMGB1, TNFα, NFKB | Carbone, 2011 (53) |
| | TNFα + crocidolite, chrysotile; macrophage + crocidolite, chrysotile | 4, 8 | 3D foci | Biopersistence of fibers via HMGB1, TNFα | Qi, 2013 (54) |
| MeT-5A, LP-9 | Carbon nanotube | 16 | Colony foci, soft agar, cell invasion | H-Ras, Erk 1/2, MMP-2 | Lohcharoenkal, 2014 (55) |
| HMC (pleural tissue) | Chlamydia pneumoniae | 2 | PCR of MPM biomarkers, cell invasion | Induced MPM markers | Rizzo, 2014 (56) |
| MeT-5A | Up chimera (disrupt DNMT1/PCNA/UHRF1 complex) | 3 | Tumor xenografts | Global DNA hypomethylation | Pacaud, 2014 (57) |

them to speculate about the role of autocrine growth signaling in tumorigenesis as well as the possibility of multiple unique pathways leading to the malignant state in MPM (48).

### *Transformation by asbestos exposure*

Early experience by Lechner *et al.* with exposing HMC to asbestos revealed that HMC were highly and uniquely sensitive to the cytotoxic effects of all asbestos fiber forms tested (60). Despite different concentrations of amosite, mice xenograft tumorigenicity experiments were unsuccessful because most exposed HMC were killed and only rare sub-populations of HMC could be passaged for further study. The few surviving cells from this experimental model did reveal that asbestos induced complex chromosomal aberrations. Thus, this early study, among other similar ones (61), established the apparent paradox of asbestos action: the killing of most HMC upon their exposure yet was the only specific agent known to induce tumorigenicity in certain HMC and ultimately cause MPM. A follow-up study by Gabrielson *et al.* exposed HMC, MPM cell lines, or previously transformed (tumorigenic) HMC to amosite and observed similar patterns of differential susceptibility to asbestos cytotoxicity, leading them to propose that tumorigenicity and asbestos resistance are independent processes that contribute to the overall process of MPM oncogenesis (62).

Using a different approach, Bocchetta *et al.* demonstrated that SV40 and asbestos act synergistically as co-carcinogens in soft agar foci formation assays of exposed HMC over a prolonged period of 6 to 8 weeks (49). Interestingly, HMC transfected with a SV40 construct expressing both of its Tag and small tumor antigen were able to transform without crocidolite exposure, but it was the combination of asbestos and SV40 that produced the most foci. This oncogenesis model was developed during the period that SV40 involvement in MPM was a popular concept. Along

 

similar assumptions, Cacciotti *et al.* demonstrated that SV40 Tag induced, in HMC, survival from asbestos-induced cytotoxicity and apoptosis via phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) signaling (51). This study helped to reinforce the hypothesis of multiple Tag-dependent survival pathways [e.g., hepatocyte growth factor/hepatocyte growth factor receptor (c-Met)] in HMC driving the selection of a sub-population able to evolve to a fully malignant state after they are transformed by asbestos (63).

### *Transformation by silicates*

Others investigated the transforming effects of alternative non-asbestos materials in oncogenic models of MPM, namely because of the known cytotoxic effects of asbestos to HMC paradoxically limiting their formation of transformed tumorigenic cells. Wang *et al.* explored the ability of combinatorial exposures of inflammatory cytokines [IL-1β and/ or tumor necrosis factor (TNF)-α] with or without erionite to induce transformation of MeT-5A cells as measured by anchorage-independent growth in soft agar assays over prolonged periods of at least 16 weeks (50). Erionite is a silicon-based asbestiform fiber belonging to the mineral class of zeolites found to be naturally occurring in rock formations throughout worldwide locations with known epidemiologic link to MPM (53). Although it is accepted that erionite is a carcinogen (64,65), the molecular mechanism(s) responsible for its toxicity remain obscure with recent recognition that iron, previously thought to be the critical element driving carcinogenicity (66), is not even part of the erionite crystal structure (67). To date, the work from Wang *et al.* is the only study to demonstrate that prolonged exposure to cytokines (IL-1β with TNF-α) could induce transformation of non-cancer cells without asbestos. Erionite exposure alone did not induce transformation, but required the additive effects of these cytokines.

Contrary results were obtained by Bertino *et al.* who induced transformation of HMC by low doses of erionite over long-term exposure, noting that this mineral fiber was poorly cytotoxic but able to stimulate pathologic proliferation via constitutive signaling of Akt, NF-κB and extracellular signal-regulated kinases (Erk) 1/2 pathways (52). Amosite and chrysotile asbestos fibers were not able to transform HMC under the same experimental conditions, but, like other studies, did produce extensive cell killing by cytotoxicity. It was claimed that these transformation results were not dependent on the presence of SV40, yet there were no confirmatory assays specifically checking for SV40.

### *Transformation by co-culture with macrophages*

Carbone *et al.* sought to clarify the biologic effects of erionite in HMC by co-culturing with macrophages in a dual chamber set-up that mimicked (proposed *in vivo* events) the process of inflammatory cell recruitment and activation at sites of fiber deposition (53). After a prolonged culture period, 3D foci developed, consistent with their interpretation of cellular transformation, in only those erionite-exposed HMC with macrophages but not in unexposed HMC with macrophages. Erionite fibers exerted effects on both HMC and macrophages culminating in a self-amplifying cascade of programmed cell necrosis and chronic inflammation. This same research group followed-up this experimental design of *in vitro* MPM oncogenesis using more combinations of co-culture conditions among HMC, macrophages, cytokines, and asbestos fiber types (crocidolite or chrysotile) (54). They showed that HMC required TNF-α to survive asbestos-induced cytotoxicity and that either fiber could transform HMC as observed in 3D foci formation assay. When HMC were exposed solely to either asbestos, they released HMGB1 and TNF-α in a fiber-density dependent manner, but no cells survived past 2 weeks and hence no transformation occurred under these conditions. Pretreating the HMC with TNF-α reduced asbestos cytotoxicity. Asbestos exposure of HMC co-cultured with macrophages, which secrete presumably enough TNF-α to protect HMC from necrosis, demonstrated transformation after about 8 weeks.

### *Transformation by other agents*

Technological advances have inadvertently developed high aspect ratio engineered nanoparticles, such as single-walled carbon nanotube (SWCNT), which share physical characteristics to asbestos fibers leading to concerns that these newly manmade materials could cause MPM (26,68). Lohcharoenkal *et al.* recently demonstrated in Met-5A cells exposed to prolonged SWCNT doses, the formation of increased and large-sized colonies in soft agar as well as invasiveness by transwell migration consistent with a transformed phenotype (55). However, LP-9 peritoneal normal mesothelial cells (69), only showed modest colony

 

formation yet did exhibit similar aggressive changes in invasiveness when induced with SWCNT. In additional functional assays of both MeT-5A and LP-9 cells, upregulated transforming protein p21 gene (H-Ras), Erk 1/2, and matrix metalloproteinase-2 (MMP-2) signaling were attributed to SWCNT induced changes. Since SWCNT continue to find wider usage in all fields of electronics, optics, energy storage, and/or alloys to name some of the major applications, this material could represent another future threat for an increase in MPM cases (70).

An alternative new concept regarding the pathogenesis of MPM that has not gained traction involves the purported role of *Chlamydia pneumoniae* as discussed by Rizzo *et al.* (56). They infected primary HMC with the bacteria and relied on induced expression of MPM biomarkers calretinin, Wilms' tumor 1, and osteopontin as a surrogate measure of cellular transformation. Aside from not providing definitive evidence of transformed cells (anchorage-independent growth assays or tumor formation in mice), the interaction, if any, with asbestos is not discussed nor is there a compelling human epidemiologic link established. Another more intriguing concept was reported by Pacaud *et al.* who showed that global DNA hypomethylation induced by the UP chimera protein disrupting a DNA methyltransferase complex deoxyribonucleic acid (cytosine-5)-methyltransferase 1 (DNMT1)/proliferating cell nuclear antigen (PCNA)/ubiquitin-like, containing PHD and RING finger domains, 1 (UHRF1), could transform MeT-5A cells to produce tumors in mice (57). This model is one of the first to demonstrate the impact of epigenetic dysregulation in tumorigenesis of HMC cells. As this study was investigating pan-cancer effects, they did not pursue a direct link of this oncogenic mechanism to asbestos effect. Epigenetic dysfunction could explain MPM cases without apparent asbestos exposure although the inciting mechanisms remain to be elucidated.

## Limitations of mesothelioma models

Over about 30 years, numerous *in vitro* oncogenesis models have been proposed that reflect the increased understanding in MPM pathobiology and incorporate recently recognized molecular mechanisms. Nevertheless, there remains missing knowledge concerning the precise steps that link asbestos exposure to the selection for resistance to cytotoxicity as the HMC integrates these multiple molecular perturbations to complete cell transformation and also in how genetic alterations accumulate to produce a committed malignant cell. These areas of ambiguity culminate in lack of consensus on a universally accepted *in vitro* model of MPM oncogenesis.

Most of these *in vitro* MPM models (*Table 1*) observed foci formation of transformed cells usually only after lengthy time intervals, ranging at least 4 to 16 weeks. The efficiency of induced cells to transform is relatively low with many cells being eliminated because of asbestos-induced cytotoxicity when the exposure conditions are not carefully calibrated. Another limit is lack of a precise definition for cell transformation, but most commonly either anchorage-independent cell growth and/or xenograft tumor formation should be demonstrated. Unfortunately, there are even ambiguous descriptions of what can constitute anchorage-independent cell growth as demonstrated in this review. While soft-agar growth is well-accepted, other assays such as monolayer foci and 3D foci remain ambiguous as to the precise growth conditions (*Table 1*). Additionally, the baseline cells are varied and none of them are truly "normal". MeT-5A cells are altered with SV40 sequences and have abnormal ploidy status due to long-term adaptation in cell culture (our unpublished data). LP-9 cells too easily senesce and, in general, grow very slowly, making cultivation of sufficient numbers of cells for ongoing *in vitro* use inconsistent. Also, there is a notion of differential biology between pleural and peritoneal HMC in responding to asbestos effects (71) that may affect interpretation of oncogenesis mechanisms. Even primary HMC cells (from fluids of the pleural or pericardial spaces) can adapt away from their native *in vivo* state as part of the thin mesothelium, possibly biasing their behavior to transformation conditions since these HMC have adapted to non-contact growth in pleural fluid unattached to the sub-mesothelial layers.

Another perplexing aspect of MPM *in vitro* models is the simultaneous requirement for inflammatory priming of non-malignant cells in the presence of asbestos. Under this condition, it is difficult to reconcile and discern the precise sequence of survival signaling since NF-κB can activate a large number of downstream genes and pathways in a cell-type and context-dependent manner (72,73). Additional insights into the pathophysiology of MPM genesis could be discerned if the specific NF-κB-activated gene networks could be identified. With inflammation and asbestos effects exerted simultaneously, this may not be an effective method to delineate detailed sequences of

 

molecular events necessary for full cellular transformation (i.e., tumorigenicity).

## Conclusions

For the foreseeable future, MPM will remain a significant worldwide malignancy without effective interventions that translate into long-term survival for the majority of affected patients. Despite caveats, *in vitro* oncogenesis models have contributed important insights for a better understanding of MPM pathobiology. There remain molecular mechanisms to be resolved in greater detail at various steps of the current accepted model of MPM development that incorporates asbestos genotoxic and mutagenic effects with inflammatory signals. It will be of great interest to see further innovation in different *in vitro* model schemes that could yield more information. Recent studies reviewed here suggest several upcoming novel insights into pathogenic mechanisms of MPM, for example, regarding the role of epigenetics and other causative agents.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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