Introduction

Malignant pleural mesotheliomas (MPM) are rare and highly lethal neoplasms attributable primarily to environmental or occupational exposure to asbestos and related fibers, influenced in some cases by predisposing germline mutations (1). Recent advances in “omics” technologies have enabled comprehensive gene and transcriptome analyses that have provided considerable insight regarding the pathogenesis, prognosis, and treatment of MPM (2,3). Presently, less information is available regarding mechanisms and clinical relevance of epigenetic derangements in MPM (4,5). This review will focus on recent advances pertaining to the epigenetics of MPM, and potential implications regarding the development of epigenetic therapies for these neoplasms.

Epigenetic regulation of gene expression

Epigenetic regulation of gene expression occurs in the context of chromatin, the basic unit of which is the nucleosome. Each nucleosome is composed of 147 bp of DNA wrapped twice around an octamer of core histones (H2A, H2B, H3, and H4). Lysine rich tails of core histones (particularly histone H3) extend out from the nucleosome, providing sites for reversible, covalent modifications such as acetylation, methylation, ubiquitination, phosphorylation, and SUMOylation that facilitate activation or repression of gene expression (6,7).

DNA methylation is the major epigenetic mechanism mediating dynamic changes in gene expression during normal cellular homeostasis and tissue differentiation, as well as long-term repression of imprinted alleles, germ cell restricted genes, repetitive DNA, and endogenous retroviral sequences (8,9). Three major DNA methyltransferases (DNMT1, 3A, and 3B) have been identified in normal somatic cells, all of which mediate transfer of a methyl group from S-adenosyl-methionine to the 5' position of cytosine in the context of CpG (10). Clusters of CpG dinucleotides (CpG islands) are located in promoters of approximately 60% of genes; most of these islands are not methylated, thereby allowing a relaxed, transcriptionally active (euchromatin) structure (11). Additional CpG dinucleotides and CpG islands are dispersed throughout the genome; these CpGs are typically hypermethylated in normal cells (11). Whereas considerable overlap exists, DNMT1 binds preferentially to hemimethylated DNA, and functions mostly as a maintenance methyltransferase, restoring DNA methylation patterns during DNA replication or repair. In contrast, DNMT3A and 3B recognize unmethylated or hemimethylated DNA and mediate
Acetylation/deacetylation and methylation/demethylation are important for maintaining chromatin structure and gene expression during normal and cancer cells (25,26). Histone acetylation is mediated by a variety of histone acetyltransferases (HATs) whereas histone deacetylation is mediated by HDACs that are divided into four classes [reviewed in ref (26-28)]. Histone acetylation increases net negative charge leading to repulsion of DNA, relaxation of chromatin, and activation of gene expression (7). Many non-histone proteins including Hsp90, SP1, p53, and HDAC1 are targets for HATs and HDACs (27-29).

Histone lysine methylation is mediated by a variety of histone lysine methyltransferases (KMTs) that mediate mono- di- and trimethylation of specific residues, whereas histone demethylation is mediated by histone demethylases (KDMs) (26,30-32). These histone modifications are highly dynamic in response to environmental signals (33,34). Unlike histone acetylation, histone lysine methylation does not alter charge of core histones. Furthermore, in contrast to histone acetylation which is always a histone activation mark, histone lysine methylation may facilitate or inhibit gene expression depending on the site. For example, methylation of histone H3K9 and H3K27 coincides with transcriptional repression; in contrast, H3K4, H3K36 or H3K79 methylation is associated with gene activation. A variety of non-histone proteins including NFκB, p53, and E2F1 are targets of KMTs and KDM (30,31).

Recently, ATP-dependent chromatin remodeling complexes (CRC) have emerged as critical mediators of epigenetic regulation of gene expression in normal and malignant cells (35,36). To date four families have been characterized including switch/sucrose nonfermentable (SWI/SNF), imitation SWI (ISWI), chromodomain helicase DNA-binding (CHD), and INO80, named for its ability to regulate inositol-responsive gene expression. These complexes have multiple subunits with various isoforms, and exhibit pleiotropic functions including regulation of gene expression, maintenance of chromatin structure, replication of pericentromeric heterochromatin, ribosomal RNA repression, and DNA double strand break repair (37). The mechanisms by which these complexes remodel chromatin vary among and within different families. For instance, SWI/SNF complexes disassemble nucleosomes to expose DNA, whereas ISWI, INO80 and CDH family members reposition (slide) nucleosomes and stretch out the intervening DNA, thereby increasing accessibility to transcription factors; these latter complexes can also assemble nucleosomes, and are thus important for maintaining chromatin structure and genomic stability (35-37).

Advances in transcriptome analysis have revealed that >90% of the genome is transcribed as noncoding RNAs (38). Particularly relevant to this discussion are recent observations that long noncoding RNAs (lncRNA) are critical mediators of chromatin structure and gene expression during normal...
cellular homeostasis and malignant transformation (39). In addition to other highly diverse activities (40), IncRNAs function as scaffolds to recruit DNMTs and histone methyltransferases to chromatin (41), thereby adding another layer of epigenetic regulation in normal cells that is perturbed in malignancies.

### Epigenetic alterations in MPM

#### Methylation-mediated silencing of tumor suppressors

Although initially thought not to contribute to the pathogenesis of mesotheliomas (42), it is become clear that epigenetic alterations are common events in this disease. Indeed, given the relatively low mutational burden of mesotheliomas (3), epigenetic perturbations may be critical determinants of malignant transformation of pleural mesothelial cells following exposure to asbestos and related fibers. Christensen et al. (43) examined promoter DNA methylation status of six genes regulating cell cycle progression in 70 MPM. Extent of methylation of these genes correlated with lung asbestos burden as well as overall survival. Goto et al. (44) used micro-array and quantitative methylation specific PCR techniques to examine methylation status of over 6,000 CpG islands in 20 MPM relative to 20 pulmonary adenocarcinomas. An average of 387 genes (6.3%) were hypermethylated in mesotheliomas compared to 544 genes (8.8%) in lung adenocarcinomas. Higher levels of DNA methylation correlated with decreased patient survival. Three genes (TMEM30B, KAZALD1, and MAPK13) were specifically hypermethylated in MPM. Numerous other reports have documented DNA methylation-mediated silencing of tumor suppressor genes in MPM (Table 1); hypermethylation of some these genes may impact survival of mesothelioma patients [reviewed in ref (4)].

Whereas accumulating evidence indicates recurrent hypermethylation of tumor suppressor genes in MPM, the mechanisms underlying this phenomenon as yet have not been elucidated. Cytokine signaling can modulate DNMT expression and mediate hypermethylation of target genes in colorectal carcinoma and erythroleukemia cells (45,46); conceivably, cytokines induced by high mobility group box 1 (HMGB1) or the NLRP3 inflammasome in response to asbestos exposure dysregulate expression and/or targeting of DNMTs and other components of the DNA methylation machinery during evolution of MPM (47-50).

Recently we performed qRT-PCR analysis of a panel of genes encoding epigenetic regulators in a panel of cultured cell lines derived from asbestos associated MPM relative to either LP-9 (a commercially available normal mesothelial cell line) or a normal mesothelial line established in our laboratory. DNMT1, DNMT3A and DNMT3B appeared to be over-expressed in the majority of MPM lines (Table 2). Consistent with these findings, TCGA data demonstrate a spectrum of DNMT expression in MPM, and suggest that over-expression of DNMT1, DNMT3A and DNMT3B correlates with shorter survival of pleural mesothelioma patients (Figure 1).

In recent studies, Kim et al. (51) used RNA-seq and methylated DNA immunoprecipitation techniques to comprehensively characterize methylation and gene expression profiles in pluripotent side populations (SP) and non-SP fractions of a human mesothelioma line. Six thousand and four hundred genes were hypermethylated, while 3,483 were hypomethylated in SP compared to non-SP fractions. Seven hundred and ninety-five genes were upregulated whereas 335 were significantly repressed in SP fractions relative to non-SP. Concomitant changes in DNA methylation and expression levels were noted for 122 genes; 118 were hypermethylated and downregulated, whereas 4 were hypomethylated and upregulated. Ten genes exhibited hypermethylation of promoter CpG islands in association with repression. Gene ontology analysis revealed significant enrichment for stem cell maintenance, stem cell development, and stem cell differentiation.

In additional studies Kim et al. (52) used micro-array
techniques to comprehensively examine microRNA (miR) expression profiles in SP and non-SP fractions of the human mesothelioma cell line used for the aforementioned DNA methylation analysis. Ninety-five miRs were differentially expressed in the SP fraction. Gene ontology analysis demonstrated enrichment for stem cell maintenance, programmed cell death, cell proliferation, cell migration, and cellular response to stress. ErbB2 receptor tyrosine kinase signaling was the most represented pathway, suggesting that similar to what has been observed in other malignancies (53,54), ErbB2 signaling is critical for maintaining pluripotency and possibly treatment resistance in mesothelioma cells.

**Loss of imprinting (LOI) and de-repression of CG genes**

DNA hypomethylation has been implicated in LOI, de-repression of endogenous retroviral sequences, and activation of CG genes which may enhance proliferation, genomic instability, and resistance to apoptosis during malignant transformation (55-57). Presently, limited information is available regarding mechanisms and clinical relevance of DNA hypomethylation in MPM. For instance, with the exception of a single report of LOI of IGF-II in

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a pleural mesothelioma inducing hypoglycemia (58), this phenomenon has not been described in MPM. Similarly, expression of endogenous retroviral sequences has not been evaluated in MPM.

Aberrant activation of CG genes [also referred to as cancer-testis (CT) genes] in somatic cells during malignant transformation results in expression of highly restricted tumor antigens that induce serologic as well as cell-mediated immune responses in cancer patients; as such, cancer-testis antigens (CTAs) have emerged as attractive targets for cancer immunotherapy (59). To date, more than 270 CG genes have been registered in the CT database (http://www.cta.lncc.br); 75% of these genes are expressed only in normal testis and malignancies, whereas the remainder exhibit high level expression in testis and variable expression in other normal tissues, and cancers (24). Approximately half of the CG genes are encoded on the X chromosome. These cancer-testis-X chromosome (CT-X) genes frequently comprise extended families with inverted DNA repeats. In contrast, the non-X CT genes are not associated with extended families or inverted repetitive DNA sequences (60,61). Relative to autosomal CT genes, CT-X genes tend to be more frequently activated in cancers, and particular gene families are coordinately up-regulated in a tumor-specific manner suggesting transcriptional co-regulation, and functional relatedness of the respective gene products.

In general, the magnitude of CG gene de-repression in human cancers coincides with advanced stage of disease, and there is mounting evidence that activation of these genes enhances the malignant phenotype of cancer cells. For example, BORIS/CTCFL up-regulates h-TERT (62), and through poorly defined mechanisms inhibits apoptosis in cancer cells (63). MAGE-A11 inhibits function of the RBL1/p107 tumor suppressor (64), and MAGE-B2 enhances E2F activity to promote cell cycle progression (65). MAGE-A2, and MAGE-C2 impair p53 function by directly inhibiting binding of p53 to target promoters, promoting deacetylation (inactivation) of p53, or by enhancing ubiquitin-mediated degradation of this tumor suppressor (66-68). Recent reports demonstrating high level CT-X gene expression in cancer stem cells (69-71), raise the possibility that CT-X antigens function to enhance pluripotency.

Although associated with genomic hypomethylation, de-repression of CG genes does not appear to be simply a manifestation of pluripotency. Loriot et al. (72) observed no up-regulation of 18 different CG genes in human ESC, mesenchymal stem cells or adipose derived stem cells. Consistent with these findings, we observed that CG genes such as NY-ESO-1, MAGE-A1, and MAGE-A3 that are commonly upregulated in thoracic malignancies, remain transcriptionally repressed in induced pluripotent stem cells (iPSC) derived from normal small airway epithelial cells (Shukla et al.; submitted). Although these findings could suggest incomplete reprogramming in iPSC, up-regulation of CG genes in cancer cells may require more extensive DNA hypomethylation, as well as tissue-specific activation of transcription factors.

Several studies have been performed to examine the mechanisms regulating CG gene expression in cancer cells. Cartron et al. (73) observed that epigenetic repression of NY-ESO-1 in mesothelioma cells was mediated by sequential recruitment of HDAC1-mSin3A-NCOR.
DNMT3b- HDAC1-Egr1, and DNMT1-PCNA-UHRF1-G9a complexes to the NY-ESO-1 promoter. Consistent with these findings, de-novo activation of NY-ESO-1 requires genetic deletion or pharmacologic inactivation of both DNMT1 and DNMT3B (74). Hong et al. (74) reported that spontaneous or pharmacologic induction of NY-ESO-1 coincides with up-regulation and recruitment of BORIS/CTCFL with displacement of CTCF from the NY-ESO-1 promoter. Kang et al. (75) demonstrated that the transcription factor specificity protein 1 (Sp1) directly interacts with BORIS/CTCFL but not CTCF, and that Sp1 is required for BORIS-mediated activation of NY-ESO1.

Recently, Cannuyer et al. (76) sought to examine mechanisms regulating activation of CG genes in melanoma cells. Analysis of transcriptomic data revealed that CG gene de-repression was not associated with differential expression of gametogenic regulators. Instead, CG gene activation coincided with repression of a set of genes regulating mitosis/cell division. This gene expression signature was similar to one previously observed in epithelial cells following depletion of DNMT1 (77). CG gene activation and downregulation of inversely correlated mitosis/cell division genes in melanoma samples was associated with a modest, but statistically significant decrease in expression of DNMT1, but did not correlate with alterations in DNMT3A, DNMT3B, TET1, TET2, TET3, or UHRF1 expression.

Presently, limited information is available regarding expression of CG genes in MPM. Sigalotti et al. (78) used RT-PCR techniques to examine expression of MAGE1-4, NY-ESO-1, GAGE1-2, GAGE1-6, SSX2, SSX1-6 and RAGE-1 in five MPM lines relative to normal mesothelial cells. Consistent with what we have previously reported for lung cancers (79), heterogeneous CG gene expression was observed in these MPM lines, with each line expressing a unique profile. Normal mesothelial cells did not express any of these genes. Consistent with our previously published data (80), CG genes including NY-ESO-1 were readily up-regulated in MPM lines by the DNA demethylating agent 5-aza-CdR (78).

To more comprehensively examine CG gene expression in MPM, we have analyzed the TCGA database focusing primarily on those genes that are normally expressed only in germ cells, and aberrantly activated in cancers, and have been shown to be regulated by DNA methylation mechanisms (24). Table 3 depicts results of 15 of the 87 mesothelioma samples in the database. Virtually all MPM exhibited de-repression of CG genes, although the patterns and magnitude of activation of these genes were quite variable. MAGE family members were the most consistently up-regulated CT-X genes, whereas BAGE2 and CAGE1 were the most commonly up-regulated autosomal CG genes activated in MPM. Several tumors exhibited extensive de-repression of CG genes, which did not appear to coincide with relative expression levels of genes encoding DNMTs, HDACs, Sp1, sirtuins, or TET proteins (data not shown).

Polycomb mediated gene silencing

Polycomb group proteins (PcG) proteins are critical determinants of pluripotency and differentiation of stem cells (81), as well as aberrant gene expression during malignant transformation (82,83). Two major polycomb repressor complexes (PRC) have been identified in mammals (83). The initiation complex, PRC-2, contains EZH1/EZH2, SUZ12, EED and RBAP46/48 subunits, and mediates trimethylation of histone 3 lysine 27 (H3K27Me3). The maintenance complex, PRC-1, containing PCAF, PHC, RING1, CBX, and BMI1 subunits, mediates ubiquitination of H2AK119 (H2AK119Ub) (82,83). These histone marks coincide with recruitment of CRC, formation of heterochromatin - frequently in the context of DNA hypermethylation, and repression of gene expression (82,83). Several proteins such as JARID2 and additional sex comb-like (ASXL) family members physically interact with EZH2 and SUZ12 to target PRC-2 to polycomb response elements (PRE) throughout the genome (84,85). Although often associated with promoter hypermethylation, polycomb-mediated gene silencing may occur independent of DNA methylation (86,87), typically in the context of bivalent chromatin, exhibiting occupancy of PcG proteins and simultaneous activation (H3K4Me3) and repressive (H3K27Me3) histone marks. Frequently observed in stem cells, bivalent chromatin maintains differentiation-related genes in a repressed, but poised state for rapid activation or permanent silencing depending on the differentiation signal (88,89).

Observations by Goto et al. (44) that a subset of genes repressed in MPM exhibited H3K27Me3 without DNA hypermethylation suggested that perturbations of polycomb gene expression might contribute to the pathogenesis of these neoplasms. To examine this issue, we used microarray, qRT-PCR, immunoblot and immunofluorescence (IHC) techniques to examine polycomb group (PcG) gene/protein expression in a panel of cultured MPM lines and normal mesothelial cells (90).
## Table 3 Expression levels of cancer-germline genes in MPM

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**MPM,** malignant pleural mesotheliomas.
This analysis demonstrated over-expression of EZH2 (both splice variants), and to a lesser extent, EED and SUZ12 in MPM cells relative to cultured normal mesothelial cells (LP3, LP9). Immunoblot experiments demonstrated over-expression of EZH2 with concomitant increases in global H3K27Me3 levels in MPM cells relative to normal mesothelial cells. qRT-PCR, immunoblot, and IHC experiments demonstrated over-expression of EZH2 in approximately 80% of primary MPMs (the vast majority of which were epithelioid histology). shRNA-mediated knock-down of EZH2 (both variants) or EED [which is critical for maintaining stability of PRC-2, and histone methyltransferase activity of EZH2 (83)] decreased global H3K27Me3 levels and significantly inhibited proliferation, migration, clonogenicity and tumorigenicity of MPM cells. The S-adenosylhomocysteine hydrolase (SAH) inhibitor, DZNep, which is known to deplete PRC-2 components (91), recapitulated the effects of EZH2/EED depletion in MPM cells in-vitro and in-vivo. Microarray with Gene Set Enrichment Analysis confirmed enrichment of polycomb targets in MPM xenografts from DZNep treated mice. Additional analysis revealed that increased intratumoral expression of either of the two EZH2 splice variants detected by Illumina array techniques correlated with decreased overall survival of MPM patients undergoing potentially curative resections (90).

Collectively, these experiments were the first demonstration that EZH2 is over-expressed in MPM, and that PRC-2 is a potential therapeutic target in these neoplasms. Subsequent analysis of TCGA has confirmed up-regulation of EZH2 in MPM (92), as well as the significant association between EZH2 over-expression and decreased survival of MPM patients (Figure 2A). Further analysis of TCGA demonstrates that SUZ12 over-expression also portends poor survival in MPM patients (Figure 2B). In contrast, there does not appear to be any association between EED expression and survival of MPM patients (Figure 2C).

The aforementioned findings are of particular relevance given recent observations that rare familial MPMs, as well as ~60% of sporadic MPMs exhibit inactivating mutations involving BRCA-1 associated protein-1 (BAP1), which encodes a nuclear ubiquitin hydrolase with diverse activities including de-ubiquitination of H2AK119Ub (93-95). BAP1 directly interacts with ASXL1, but not EZH2 or SUZ12 (85). As previously mentioned, ASXL1 interacts with EZH2 and SUZ12 to recruit PRC-2 to DNA (85). As such, the ASXL1-BAP1 complex may function to mitigate the repressive activities of ASXL1-PRC-2. Collectively, these findings suggest that perturbations of the BAP1-ASXL1-polycomb repressor (BAPR) axis are central themes of mesothelioma development. Whereas the effects of BAP1 mutations on gene expression in MPM have been described (93), the epigenomic effects of BAPR dysregulation in these neoplasms have not been evaluated in a comprehensive manner. Conceivably, global and promoter-specific PRC-2 marks, DNA methylation, microRNA and gene expression profiles, as well as responses to biochemical or pharmacologic inhibition of PRC-2 activity may be contingent on BAP1 mutation status as well as magnitude of EZH2 over-expression in MPM.

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Figure 2 Association of intra-tumoral expression of genes encoding core components of PRC-2 and survival of MPM patients. Kaplan Meier curves demonstrating that the magnitude of EZH2, and SUZ12 (A and B, respectively), but not EED (C) detected by RNA-seq techniques adversely impacts patient survival. No association was observed for ASXL1 expression and survival (data not shown). MPM, malignant pleural mesotheliomas.
In a series of elegant experiments, LaFave et al. (92) observed that BAP1 mutations, which typically result in loss of protein expression, increased EZH2 as well as SUZ12 expression in MPM cells. Up-regulation of EZH2 in BAP1 mutant cells was associated with reduced levels of H4K2Me1, as well as decreased occupancy of L3MBTL2 (an atypical polycomb protein which recognizes this repressive histone mark) within the EZH2 promoter (96,97). Additional experiments demonstrated that BAP1 deubiquitinates and thereby stabilizes and co-localizes with L3MBTL2 within the EZH2 promoter. BAP1 mutant MPM cells exhibited decreased H4K20Me1 in the EZH2 promoter; presently it is not known if this was due to a decrease in expression/activity of SETD8, the KMT that catalyzes mono-methylation of H4K20 (30), or up-regulation of an unidentified histone demethylase. BAP1 mutations decreased occupancy of BAP1 as well as L3MBTL2 within the EZH2 promoter. Relative to MPM cells expressing BAP1, MPM cells with BAP1 mutations were markedly more sensitive to pharmacologic inhibitors of EZH2 in-vitro and in-vivo (92), suggesting that BAP1 mutations render MPM cells addicted to PRC-2.

Despite strong association between BAP1 mutations and repression of stem cell polycomb targets (93), there does not appear to be a unique clinical phenotype of BAP1 mutant MPM. Interestingly, current or former smokers with MPM appear more likely to have somatic BAP1 mutations, although nucleotide substitutions do not suggest a direct causal role of smoker exposure and BAP1 mutations (98). Additionally, in contrast to EZH2 or SUZ12 over-expression which appears to be associated with decreased patient survival, BAP1 mutations appear be associated with improved patient survival despite up-regulation of both of these PRC-2 components (99,100). This paradox may be related to deficient BRCA-1-mediated DNA repair, which may render BAP1 mutant MPM cells more sensitive to DNA damaging agents. To date however, there have been no published studies examining mutational loads in BAP1 mutant vs BAP1 wild type MPM.

Additional studies have been performed to examine mechanisms contributing to EZH2 over-expression that potentially could be targeted therapeutically in MPM. In silico analysis of the EZH2 promoter revealed numerous recognition elements for SP1, a zinc-finger transcription factor over-expressed in a variety of human malignancies (101). qRT-PCR, immunoblot and IHC experiments demonstrated markedly higher SP1 expression levels in MPM lines and primary MPMs relative to cultured normal mesothelia or normal pleura. Over-expression or knock-down of SP1 significantly increased or decreased EZH2 expression, respectively; furthermore, knock-down of SP1 diminished proliferation of MPM cells (102).

**SWI/SNF**

CRC: SWI/SNF complexes- mammalian homologs of yeast trithorax, function to antagonize the repressive activities of PRC-2 in part by disrupting DNA-nucleosome contacts, and facilitating movement, ejection, or substitution of nucleosomes to enhance transcription factor accessibility to DNA (103,104). SWI/SNF complexes are composed of 10–15 subunit multimers encoded by 29 genes. Genes encoding SWI/SNF complexes rank among the most frequently mutated genes in human cancers, with specific subunit mutations being associated with unique cancer histologies (103,104). Yoshikawa et al. (105) performed whole exome sequencing on short-term lines established from 8 MPM, and noted significant enrichment for mutations in genes involved in SWI/SNF pathways, including homozygous mutations of SMARCA4, ARID2, and PBRM1; one patient had homozygous germline mutations in SMARCC1 and SETD2, a histone methyltransferase that catalyzes formation of H3K36Me3 (activation mark). More recently, Yoshikawa et al. (2) used high-density array comparative genomic hybridization (a-CGH) and targeted next-generation sequencing (NGS) techniques to examine somatic copy loss in the 3p21 region (approximately 10.7 Mb containing 251 genes) in 33 MPM. Minute (<3 Kb) bi-allelic deletions were detected in 46 genes; 4 of which have been associated with malignancies, including two SWI/SNF related genes [PBRM1 (15%) and SMARCC1 (6%)], BAP1 (48%) and SETD2 (27%). In a recent comprehensive genomic analysis of over 200 MPM, Bueno et al. (3) reported no mutations involving genes encoding SWI/SNF components, but did observe SETD2 mutations in 8% of specimens, as well as mutations involving two additional histone methyltransferases (SETDB1 and SETD5) in approximately 3% of specimens. Discrepancies between results reported by Yoshikawa and colleagues (2) and Bueno et al. (3) may be attributable to identification of minute deletions by high-density a-CGH and targeted NGS that are not detectable by conventional NGS techniques. Additional studies are required to more fully interrogate the frequency and clinical implications of SWI/SNF mutations.
Epigenetic strategies for mesothelioma therapy

Aforementioned data demonstrate that similar to other cancers (11), MPM exhibit silencing of tumor suppressor genes via site specific DNA hypermethylation and/or polycomb repressive complexes in the context of genome wide hypomethylation that facilitates LOI and de-repression of CG genes. This “DNA methylation paradox”, recapitulates epigenomic states in normal germ cells, and provides the rationale for the development of epigenetic regimens that induce growth arrest/apoptosis via restoration of tumor suppressor gene expression (106-108), while simultaneously augmenting antitumor immunity by up-regulation of CTAs (79), induction of viral mimicry by de-repression of endogenous retroviruses (109,110), and modulation of the tumor microenvironment (111-113).

Given their direct roles in silencing tumor suppressor genes and maintaining pluripotency (114,115), DNMTs are attractive targets for MPM therapy. However, previous clinical efforts to inhibit DNMT activity in MPM have been disappointing. Yogelzang et al. (116) reported a 17% objective response rate in 41 MPM patients receiving continuous 120 h dihydro-5-azacytidine infusions; interestingly, the one complete responder was free of disease six years following treatment. Schrump et al. (117) observed transient stabilization of disease in 2 of 6 MPM patients receiving continuous 72 h decitabine infusions.

The lack of efficacy of DNA hypomethylating agents in solid tumors to date may be related to the fact that these agents have been dosed to maximum tolerated levels resulting in myelosuppression rather than administered chronically at lower doses to achieve pharmacodynamic effects without systemic toxicities. Data from our phase I decitabine (DAC) trial clearly demonstrate that chronic exposures are required to achieve maximal gene induction effects in cancer tissues (117). Furthermore, the short half-lives (<5 min) and poor biodistribution of 5-AZA and DAC administered by IV, SQ or PO routes limits their potential use in patients with solid tumors. These compounds are rapidly inactivated by cytidine deaminase (CDA) which is present in virtually all organs—particularly those in the GI tract (118). Recent studies in nonhuman primates (119) as well as a phase I trial in patients with sickle cell disease (Saunthararajah et al.; submitted) have demonstrated that oral tetrahydrouridine [an inhibitor of CDA that has been administered intravenously to thousands of cancer patients without documented toxicities (120,121)] significantly increases Cmax and t1/2 (>50 nM and 4 h, respectively) and increases biodistribution of oral decitabine thereby significantly decreasing interpatient variability regarding drug levels; oral DAC-THU mediated systemic DNA hypomethylation evidenced by significant increases in fetal hemoglobin, without neutropenia, thrombocytopenia or lymphopenia. A phase II trial (NCT02664181) is presently underway at the Cleveland Clinic and NCI to examine if DAC/THU can improve responses to nivolumab when administered as second line therapy to patients with non-small cell lung cancers (NSCLC). A phase I/II dose escalation trial will commence at the NCI in the very near future to ascertain if oral DAC/THU can enhance responses to pembrolizumab when administered as first line therapy for NSCLC with high PD-L1 expression. If positive, results of these trials would support evaluation of similar regimens in mesothelioma patients (see below).

Despite encouraging preclinical data (122), efforts to target HDACs in MPM have been discouraging as well. Krug et al. (123) randomized 661 MPM patients to receive the HDAC inhibitor, vorinostat or placebo as 2nd or 3rd line therapy. Primary endpoints were overall survival (OS) as well as safety and tolerability of vorinostat. Median OS for vorinostat treated patients was 30.7 weeks (95% CI: 26.7–36.1) compared to 27.1 weeks (95% CI: 23.1–31.9) for patients receiving placebo. The lack of efficacy of single agent vorinostat in MPM patients is not surprising given little evidence of over-expression of HDACs in MPM (Table 2), and rather limited antitumor effects of HDAC inhibitors alone in preclinical experiments (124). Combination strategies such as the use of HDAC inhibitors to sensitize cells to TRAIL mediated apoptosis (125), or the use of flavopiridol to enhance romidepsin mediated growth arrest and apoptosis (124) might be appropriate to evaluate in future clinical trials.

Given the frequency and negative prognostic impact of EZH2 over-expression in MPM (90,92), PRC-2 has emerged as a major therapeutic target in these neoplasms—particularly those with BAP1 mutations. Whereas DZNep is not available for clinical trials, several potent and specific inhibitors of EZH2 activity are in early clinical development. A multicenter phase II trial (NCT02860286) is underway to examine response rates in patients with inoperable, BAP1 mutant MPM treated with oral tazemetostat (800 mg BID). A two arm phase II trial will commence in the near future at the NCI to examine response rates in patients with wild type vs mutant
BAP1 MPM receiving GSK126 as induction therapy prior to pleurectomy/decortication; a variety of translational endpoints will be assessed in this trial. Additionally, mithramycin, which depletes EZH2 as well as several other PRC-2 associated proteins (102), is being evaluated in patients with inoperable thoracic malignancies (including MPM) at the NCI (NCT01624090, NCT02859415).

It may be possible to further exploit BAP1 mutations for MPM therapy. BAP1 functions to stabilize BRCA-1 and promote poly(ADP-ribose)-dependent recruitment of polycomb deubiquitylase complex PR-DUB to DNA damage sites (126,127). This activity is dependent on deubiquitinase activity as well as phosphorylation of BAP1 (128). BAP1 mutations, which always appear to be manifested as loss of function, decrease BRCA-1 levels (129), and inhibit double strand DNA repair (126-128). Parotta et al (130) observed that a BAP1 isoform lacking part of the catalytic domain sensitized MPM cells to the PARP1 inhibitor, olaparid; and this sensitivity could be augmented by concomitant treatment with the dual PI3K-mTOR inhibitor, GDC0980, which downregulates BRCA-1. Such strategies might enhance responses to cisplatin/pemetrexed in patients with BAP1 mutant MPM, and should be evaluated in future clinical trials.

Given extensive preclinical studies demonstrating that DNA demethylating agents, HDAC inhibitors and KMT inhibitors mediate potentially significant immunomodulatory effects (111), there is considerable interest in utilizing chromatin remodeling agents in conjunction with either adoptive cell transfer or immune checkpoint inhibitors for cancer therapy. Previously we demonstrated that a cancer testis antigen upregulated in vivo by decitabine could be targeted by cytolytic T cells to eradicate metastatic cancer in a syngeneic murine tumor model; these experiments established the preclinical rationale for combining gene induction regimens with adoptive immunotherapy for cancer (131). Recently, Corve et al. (132) evaluated the potential efficacy of combining a gene induction regimen with anti-CTLA 4 therapy in MPM. Consistent with results of a recent phase II double blind, placebo controlled trial demonstrating no efficacy of Tremelimumad in MPM patients (133), the murine anti-CTLA4 Mab 9H10 did not significantly inhibit growth of MPM xenografts. 5-azacytidine (5-AZA) induced a slight but insignificant reduction in growth of MPM xenografts. In contrast, combined 5-AZA/9H10 treatment mediated an 81% inhibition of MPM xenograft growth (P<0.05). This phenomenon coincided with up-regulation of the murine CTA, P1A, as well as increased class I HLA expression. Collectively, these data support evaluation of DNA demethylating agents in combination with immune checkpoint inhibitors in MPM. Despite evidence that 5-AZA augmented responses to anti-CTLA therapy in preclinical models, a better translational strategy might be to combine DNA demethylating agents with pembrolizumab given recent observations that this PD-L1 inhibitor mediated a 17% objective response rate in MPM patients (134), and high levels of PD-L1 expression in MPM-particularly sarcomatoid subtypes (3). Observations that combined decitabine/GSK126 or 5-AZA/entinostat treatment markedly augment efficacy of adoptively transferred CTL or anti-PD-L1 via up-regulation of Th1 signaling and inhibition of immunosuppressive myeloid derived suppressor cells within the tumor microenvironment in murine cancer models (113,135) support evaluation of such combinatorial regimens in clinical settings. These findings, together with recent observations that the immune microenvironment impacts outcome of patients with MPM (3,136) provide compelling rationale for combined epigenetic-immunotherapies for these neoplasms.

Conclusions
Due to their recalcitrance to conventional treatment modalities, MPMs continue to challenge clinicians. Recent insights into epigenetic mechanisms which dysregulate gene expression in MPM, together with novel potent, and potentially efficacious regimens targeting DNMTs, EZH2 and PARP1, as well as immune checkpoints provide new opportunities to target the epigenome for the treatment and possible prevention of MPM.

Acknowledgements
None.

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

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