1. ABSTRACT:

Malignant mesothelioma (MM) is an aggressive cancer of the mesothelial lining of pleura, peritoneum and other body cavities. It has been associated with long term exposure to asbestos and erionite. It is a relatively rare, but deadly disease and current therapies have still a very poor clinical outcome. This thesis presents two sets of data through which different aspects of malignant mesothelioma were explored. The first study was performed by the methylation-based HUMARA assay on a panel of primary MM tumors, to identify the clonal pattern of MM. The results indicate the polyclonal origin of the MM tumors analyzed (n = 6). The second study was focused on Ca\(^{2+}\) and Mg\(^{2+}\) permeable ion channels, Melastatin Related Transient Receptor Potential 2 (TRPM2) and Melastatin Related Transient Receptor Potential 7 (TRPM7), to investigate the role of these channels both in the transformation of primary human mesothelial cells (HM) upon exposure to asbestos fibers and in motility and survival of MM cells. A time- and dose-dependent correlation between TRPM2 and TRPM7 mRNA levels and cell exposure to asbestos fibers was observed. However, while TRPM2 protein was expressed at different extents in both HM and MM cells, the TRPM7 protein was present in all MM cells but in none of the HM primary cultures tested. Moreover, in MM cells infected by lentiviral particles encoding shRNA targeting TRPM7, no differences were observed in proliferation, wound healing and migration rates, compared to scramble shRNA transduced cells. On the contrary, in MM cells transduced with shRNA targeting TRPM2, the same cancer hallmarks were significantly higher than in uninfected cells or in controls infected with scramble shRNA. In summary, the results indicate that TRPM2 has a protective role against HM transformation upon asbestos exposure and influences the maintenance of the transformed phenotype of MM cells, possibly affecting the mechanisms of cell death.
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3. LIST OF ABBREVIATIONS

HUMARA : Human Androgen Receptor Gene/Protein/Assay

HUMARA-MSP : Methylation Specific Primers Based HUMARA Assay

PCR : Polymerase Chain Reaction

TRPM2 : Melastatin-like Transient Receptor Potential 2

TRPM7 : Melastatin-like Transient Receptor Potential

shRNA : Short Hairpin RNA

DNA : Deoxyribonucleic acid

RNA : Ribonucleic acid

WB : Western Blotting

HM : Human Mesothelial Cells

MM : Malignant Mesothelioma

DMEM : Debecco's Modification in Eagle's Medium

FBS : Fetal Bovine Serum

DMSO : Deoxymethyl Sulfoxide

HMGB1 : High Mobility Group Box 1

SV40 : Simian Virus 40

ROS : Reactive Oxygen Species

EGTA : Ethylene Glycol Tetraacetic Acid

LCM : Laser Capture Microdissection
4. BACKGROUND:

4.1. Malignant Mesothelioma

Malignant mesothelioma (MM) is a highly aggressive cancer that arise from the serous membranes of the pleura, peritoneum and occasionally in the pericardial and tunica vaginalis testis cavities. It originates from the primary mesothelial cells (HM) locally, but it spreads rapidly to occupy most of the visceral and parietal surfaces. Epidemiological data and experimental studies have established the association of exposure to asbestos fibers and MM. MM pathogenesis has also been linked to: i) Simian Virus 40 (SV40) infection, ii) exposure to erionite fibers, iii) genetic susceptibility and iv) radiation. MM causes 3000 deaths per year in the US, whereas in Western Europe this number reaches to 5000 deaths in a year.

4.1.1. Etiology and Pathogenesis of MM

Fibers: Asbestos and Erionite

Asbestos is a class of silicate minerals including different types, grouped in serpentine and amphibole asbestos. Primarily the amphiboles crocidolite and amosite were recognized as the predominant cause of MM and the latency period between asbestos exposure and the development of the disease ranges from 21 to 71 years. The mechanisms of carcinogenesis in humans were recently elucidated, by showing that the main effect of asbestos fibers is the induction of programmed cell necrosis, leading to the release of HMGB1 and other pro-inflammatory cytokines. Other studies revealed that asbestos carcinogenesis can be linked to the activation of the AP-1 pathway or the Akt pathway. In addition, mutagenic oxygen radicals released mainly by lung macrophages may contribute to asbestos carcinogenesis. The most complete mechanistic characterization was performed by Yang et al., who linked TNF-α and NF-κB to asbestos pathogenesis and MM. More recently, the mechanistic model for asbestos carcinogenesis was expanded, identifying the link through the effects of the chronic inflammatory reaction by the HMGB1 release in MM (Figure 1).

Erionite, an asbestos-like type of fiber, is considered the most carcinogenic mineral fiber inducing MM, as shown by MM occurrence in almost all rats exposed to erionite inhalation, as compared to a far lower percentage (20%) of rats exposed to crocidolite or to other asbestos fibers. Erionite is present in many regions of the world. However, with the exception of Turkey, its contribution to the incidence of MM was not fully evaluated yet, until a recent study revealed that the environmental contamination of erionite in North Dakota Dunn county (US) is similar to that found in Cappadocia (Turkey), both in term of erionite chemical-physical composition and in term of intensity of fiber air dispersion. Moreover, the in vitro transforming potential of both minerals has also been found similar, highlighting the risk of erionite exposure in US.
Cofactors: SV40

Simian Virus 40 (SV40) infection has also been linked to MM pathogenesis. The relationship between this virus and mesothelioma is still not well understood, as humans are not the natural hosts for SV40. A causative association between SV40 and human cancers, including MM was proposed and has been the subject of some controversy. Variability in SV40 detection methods, lack of epidemiologic evidence linking polio-vaccinated cohorts with higher incidence of tumors and also the fact that SV40 was not found in MM in certain countries may account to some of these controversies. However, millions of people worldwide were injected with the inactivated (Salk) and early live attenuated (Sabin) forms of polio vaccines that were contaminated with SV40. Intrapleural injection of SV40 has produced mesothelioma in 100% of hamsters demonstrating its oncogenicity. SV40 Large T antigen (Tag) binds and inactivates essential tumor suppressor genes, like p53 and pRb, stimulates Met, Notch-1 and telomerase activity. More recently it has been demonstrated that Tag binding to p53 leads to the formation of a transcriptionally active complex, responsible for IGF-1 expression and cell transformation. The SV40 small t antigen (tag) also plays a role by complementing the role of Tag in the cytoplasm. It functions by binding to and inhibiting the PP2A, which indirectly reinforces mitogenic extracellular stimuli. Finally, SV40 and crocidolite asbestos have been shown to be cocarcinogens and in the presence of SV40, significantly low amounts of asbestos are adequate to induce MM. These findings indicate that the risk of MM among asbestos exposed individuals is high because of their genetic background or because of the
interactions with carcinogenic SV40. Altogether, considerable evidence supports a role of this oncogenic virus in mesothelioma pathogenesis.

**Genetic Predisposition**
Several studies indicate that mineral fiber carcinogenesis can be influenced by genetic predisposition and may lead to the development of MM. This was demonstrated in Turkish families during a true MM epidemic in Karain, Sarihidir and Tuzkoy villages of Cappadocia. In these villages about 50% or even more deaths are caused by MM, linking this epidemic to the mineral fiber erionite, a fibrous zeolite commonly found in the stones of the built homes of Cappadocia. In these families, it appears that exposure to minimal amounts of erionite is sufficient to cause MM. It was noticed that MM was more frequent in certain families compared to others, revealing the unusual susceptibility to erionite carcinogenesis in these families. Furthermore, pedigree analysis of families who lived in homes where mesothelioma occurred showed that the disease can be inherited in an autosomal dominant pattern. Approximately 50% of descendants of affected parents developed mesotheliomas. When members of unaffected families married into affected families, 50% of their descendants also developed mesothelioma. These data suggest that genetics is a key factor also because mesothelioma does not develop in non-affected families, regardless of environmental exposure. Although any link between genetic background and mesothelioma has not yet been found for Turkish families, in a recent study we discovered such a link in U.S. families with a high incidence of mesothelioma. In this study, we focused on two U.S. families; one in Wisconsin (W) and one in Louisiana (L), in which the members exposed to erionite had no history of occupational exposure to asbestos. Despite the lack of the confounding factor of heavy exposure to carcinogens known to cause a high incidence of mesothelioma, family members developed a variety of malignancies in which MM was predominant. Searching for the predisposing genetic factor, we discovered germline mutations in the gene encoding the BRCA-1 associated protein 1 (BAP-1).

**Radiation**
Radiation exposure has also been linked to MM, even though these cases are rarely observed. Patients who received radiation treatments, specifically in the thoracic or abdominal regions, have shown increased risks in developing MM. The average time between radiation exposure and the development of MM is about 21 years. Moreover, studies in rats demonstrate that radiation is a causative co-factor of MM in combination with asbestos exposure.

**Conclusion**
The overall features shown by advanced mesothelioma suggest complex and diverse patterns of changes in growth regulation during carcinogenesis, which involve both inactivation of Tumor Suppressor Genes (TSGs) and activation of proto-oncogenes. This process may be facilitated by SV40 large T antigen, which inhibits p53 leading to impaired DNA repair and apoptosis in erionite or asbestos damaged cells.
Genetic factors also have the potential to influence the carcinogenesis of human mesothelial cells under the effect of carcinogenic mineral fibers.

Figure 2: Mesothelioma pathogenesis model.

4.1.2 Classification and Morphology

Pleural mesothelioma (MM) is the most common type of mesothelioma, accounting for about 70% of all MM cases. MM is subtyped into three forms, according to the histological morphology: epithelioid, biphasic and sarcomatoid (Figure 3). The prognosis of MM is poor, and the median survival time for these three types is 18, 11 and 8 months, respectively.
Figure 3: Histologic Subtypes of MM.
(A) Epitheliod, (B) Sarcomatoid, and (C) Biphasic phenotypes

Epitheliod MM displays an epithelial-like phenotype, appearing relatively uniform, and is described as having a tubular papillary structure. Each individual cell is cube-shaped and has an easily identifiable nucleus. It is the most common form of the disease, occurring at about 50% to 70% of all diagnosed MM. However, it is considered the least aggressive and most responsive to treatments, with the best prognosis.

Sarcomatoid MM is a spindle cell type of tumor, with elongated nuclei. This cell type represents 7% to 20% of cases diagnosed. It is the most aggressive subtype and typically it does not respond to any treatment and it is the MM with the poorest prognosis.

Biphasic MM is a combination of mixed cell types, both epithelial-like and sarcomatoid located in the same tumor. The biphasic MM is identified in 20% to 35% of MM cases, and its prognosis is intermediate between the other two cell types.

4.1.3. Current Treatments

There are no therapeutic standards for MM and the treatment options depend on performance status, pulmonary function, stage, and age of the patient.

Surgery
Surgical removal of the lung (extrapleural pneumonectomy), has led to median survival time of more than 2 years; however, requiring very invasive removal of the gross tumor. Some form of adjuvant therapy aiming at eliminating residual microscopic disease typically follows this procedure. However, only few patients are suitable for this line of surgery due to complications.

Chemotherapy
Despite that numerous chemotherapy regimens have been tested, no curative chemotherapy has been found for MM so far. Two regimens are the present gold standard: Pemetrexed-Cisplatin and Gemcitabine-Cisplatin. Pemetrexed plus cisplatin is a combination of an antifolate and a platinum compound. A phase III study consisting of 448 patients showed for the combination an improvement in overall survival of about 3 months and 41% response rate, compared with cisplatin alone. Gemcitabine plus cisplatin has also been shown to have similar responses of palliation, offering symptomatic improvement, quality of life and a response rate of about 48% in a total of 74 patients. Moreover, targeted therapies have also been explored. Clinical trials were conducted with imatinib mesylate and with gefitinib to inhibit the Platelet-Derived Growth Factor (PDGF) and the Epidermal Growth Factor (EGF) pathways, respectively, but no evident response has been observed. Agents that inhibit angiogenesis pathways are currently under investigation, considering the fact that MM tumors often tend to have high microvessel density, which has been linked with poor outcome, however they have shown only limited activity until now. Phase II studies were conducted using monoclonal antibody against Vascular Endothelial Growth Factor (VEGF), Bevacizumab, or small molecule inhibitors of the VEGF receptor kinases, Sorafenib and Vatalanib. A very low effect has been observed, not meeting the criteria for further investigation. Novel anti-cancer agents such as Histone Deacetylase inhibitors (HDAC) known to be pro-apoptotic are being utilized for MM phase I trials.

The current standard of care for first line palliative chemotherapy is the combination of pemetrexed and cisplatin and with prophylactic dexamethasone plus supplemental folic acid and vitamin B12 in patients with good performance status. The most routinely used second line drugs are gemcitabine plus cisplatin.

**Radiotherapy**

Besides local postsurgical radiotherapy, studies in MM have been disappointing over the last 30 years. The diffuse nature of the tumor on pleural surfaces makes it difficult to treat by radical radiotherapy without causing pneumonitis. Most recently and successful to control disease locally, has been the use of intensity modulated radiotherapy after extrapleural pneumonectomy, where accurate placement of markers have been emphasized. However, most patients die of metastatic disease.

**Gene Therapy**

Two gene therapy approaches have been explored for MM. A suicide gene therapy involves the transfer into tumor cells DNA encoding herpes simplex virus thymidine kinase that, upon ganciclovir administration, generates toxic metabolites that destroy the tumor cells. The second, immunomodulatory gene therapy, involves the local delivery of a vector (i.e., vaccinia virus) producing a cytokine (i.e., interleukin-2 (IL-2) within the tumor over a prolonged periods of time mimicking immune responses that occur in organs undergoing autoimmune destruction). However, so far gene therapy has not been included in the standards of MM therapy.
Immunotherapy

Patients with MM usually mount an anti-MM immune response, albeit not sufficient to destroy the tumor. Previous studies with BCG (Bacillus Calmette-Guerin) vaccine documented little boost with improved survival rate in 30 MM patients. Moreover, cytokine therapy with IL-2 or interferon gamma (IL-γ) into the pleural cavity, promoted responses in patients with early disease but were ineffective with advanced disease. Furthermore, interferon alpha (IFN-α) or recombinant Granulocyte-Macrophage Colony Stimulating Factor (GMCSF) as single treatments, induced partial response rates of about 10-15%. Also, based on the preclinical models of tumor antigen mesothelin coupled to Pseudomonas exotoxin Phase I trials, dendritic cell vaccine immunotherapy were also initiated.

Despite to a number of different approaches that have been shortly described above, mesothelioma, one of the most aggressive forms of cancer, is not yet to be cured. Understanding more about the initiation of the tumor development and the factors that trigger it is crucial to have the best way to treat or even prevent this cancer. A closer look to the cell population of the tumor and exploring its progressive features might provide us with the necessary insight to approach to the tumor with the aim of eliminating it.

Given the fact that a cell population could possibly be a mixture of slightly different individual cells, any single method for the treatment may embrace the chance of the failure, since these differences might affect the efficiency of the treatment, because of the differences of drug sensitivity of the single cells. Therefore, by investigating the homogeneity of a tumor population one can answer to the fundamental question that should be considered for any possible treatment. The most common method to investigate whether a tumor population is homogenous or not is to determine its clonal origin. This approach explores the very nature of tumor initiation and categorizes the tumor as mono- or poly-clonal. A monoclonal tumor refers to a cell population derived from a single cell thus suggests a virtually homogenous population while a polyclonal tumor is known to be initiated by division of multiple differentiated cells, accordingly picturing a heterogenous population. Categorization of a cell population as mono- or polyclonal is made possible by the determination of the inactivated X chromosome of the cells in a given population. The natural event of X chromosome inactivation occurs in all female cells during the early embryogenesis and provides a sufficient tool for tracking a population to their ancestral stage, because once it is determined, the same X chromosome is kept inactivated during the mitosis of the same cell.

4.2. X Chromosome Inactivation
During early female embryonic development, one of the X chromosomes (or more accurately, most of the genes on that X chromosome) is inactivated in each cell. The subsequent progeny of each cell maintain the same inactivated X chromosome, resulting in an organism that is a mosaic of cells expressing genes from one X chromosome or the other (Figure 4).

**Figure 4**: X-chromosome inactivation and clonal development with further subclone evolution. Early in development, both X chromosomes are expressed. Before hematopoietic lineage differentiation, either of the two X chromosomes may be inactivated. This random choice of inactivated X chromosome is retained through subsequent mitoses. Oval white Xp Xm: early embryonic cells expressing both maternal and paternal X chromosomes; round Xp or Xm, nonclonal cells expressing a single X chromosome. Clonal populations can acquire multiple genetic abnormalities; each can be used to distinguish subpopulations which remain clonal. Round gray Xp: clonal cell with active paternal X chromosome; oval dark gray Xp: clonal cells with active paternal X chromosome and new somatic mutation (+).

In early 60's, Mary Lyon proposed X-chromosome inactivation (XCI) by hypothesizing that the single Barr body X chromosome came randomly from one or the other parent, rather than constantly from the father and that this chromosome was genetically inactivated. She based her hypothesis on the observations of
X-linked coat color mutations in heterozygous female mice. In these mice, the phenotype was always a mosaic, consisting of patches of normal or mutant color, rather than a homogenous blending, suggesting that early in development, in the pigmented cells either one or the other X chromosome was inactivated. Thus, if the X chromosome carrying the mutant allele was inactivated, the patch was of normal color, whereas if the X chromosome carrying the normal allele was inactivated, the patch was of mutant color. Beutler and colleagues formulated the XCI hypothesis using studies of the human X chromosome glucose 6-phosphate dehydrogenase (G6PD) gene. They found that, in females, G6PD activity was not twice as much that of males, as expected by the presence of two X chromosomes, and postulated a dosage compensation mechanism. In females heterozygous for G6PD deficiency, dosage compensation results in G6PD expression at half the rate of normal hemizygous males. This could be attributable to either half-level activity in all cells or normal expression in some cells and low expression in other cells, resulting in overall half-level expression. Using a mixture of male cells with deficient G6PD activity and normal G6PD activity, Beutler and colleagues measured G6PD activity (by glutathione stability) and compared it with the response of female erythrocytes. They found that the response curves of the 2 samples were similar in shape and concluded that intermediate activity in females was probably attributable to the same mechanism as in the mixture of male normal and G6PD activity-deficient erythrocytes.

### 4.2.1. Mechanism of X-chromosome inactivation

The exact molecular mechanisms underlying XCI are still not fully clarified, but involve several steps, including the determination of the number of X chromosomes per cell, selection of either the paternal or maternal X chromosome for subsequent inactivation, and initiation of the actual inactivating process. It has been demonstrated in mice that there are 3 non-coding loci, located near the center of inactivation of X chromosome that play a pivotal role in the mechanism of X-chromosome inactivation. These loci are: non-coding RNA X (inactive)-specific transcript (Xist), its antisense partner Tsix, and the intergenic locus Xite. Xist is necessary for cis inactivation of the X chromosome. In vitro, Xist is able to silence also the autosomal surrounding chromatin in case of X:autosome translocation, but in an incomplete manner, due to instability of autosome inactivation. Tsix and Xite work in parallel to Xist by maintaining X-chromosome transcriptional competence. Although the functions of these 3 loci have been deduced using complementary cell lines, the actual physical interactions of these components are less well known. Xist is proposed to achieve cis-inactivation of the X chromosome through close interactions between its RNA transcript and the segment of X chromosome to be inactivated. The putative trans-interactions, based on the need to determine one X to be exclusively activated and the other X to be exclusively inactivated, remained elusive, until the recent demonstration that the 2 X chromosomes undergo inter-chromosomal pairing. It is remarkable that inter-chromosomal pairings typically occur in germ cells undergoing meiosis, rather than in somatic cells undergoing mitosis.
X-chromosome inactivation timing is crucial to the interpretation of X chromosome inactivation pattern (XCIP)-based clonality assays. It has been assumed that pre-blastocyst embryos express both X chromosomes and that inactivation did not occur until after implantation and the embryonic stem cells began to differentiate into separate cell lineages. Recent experiments, however, demonstrate that XCI occurs as early as the 4-cell stage of the embryo, but is variable and leaky and does not become stabilized until after implantation, but before differentiation of embryonic stem cells into the various cell lineages. XCI before cell lineage differentiation is crucial for the interpretation of XCIP clonality studies. Hematopoietic cell lines derive not from a single embryonic stem cell but from several progenitors, allowing for the mosaic expression of genes from both X chromosomes.

4.2.2. X-chromosome inactivation–based clonality studies

In human females, the hematopoietic organ is a mosaic of 2 populations. Each population expresses alleles from one of the active X chromosomes and the choice of which X chromosome is functional is retained mitosis after mitosis. Cell lineage can be traced and subpopulation clonality can be determined by following the activated X chromosome. X chromosome protein products, transcribed mRNA, and DNA methylation status are used to determine which X chromosome is active. Protein isoforms and DNA polymorphisms are used to identify the X chromosomes. These markers must be heterozygous to differentiate between the maternal or paternal origin of the X chromosome. Thus, XCIP clonality assays are limited to females with informative markers. The first clonality studies using X-chromosome inactivation were based on G6PD isoenzymes. In the African population, there are 3 main G6PD isoenzymes: G6PD A+ and A− polymorphic variants, with faster electrophoretic mobility than wild-type G6PD B. Approximately 35% of African women are heterozygous for the electrophoretically distinguishable isoenzymes. The use of G6PD as a marker of X-chromosome inactivation is limited to females who are heterozygous for these G6PD alleles. Informative females have the A+ or A− allele on one chromosome and the wild B allele on the other.

An alternative from using protein isoforms to identify the active X chromosome and its parental origin is to directly assay the transcribed mRNA products of the active X chromosome. The parental origin of the activated X chromosome is identified by DNA polymorphisms. The exon sequence polymorphisms used to identify the X chromosome are typically non-synonymous mutations. A variety of genes are used to determine clonal status, including G6PD40 (using a polymorphism different from that used in protein-based clonality assays), iduronate-2-sulfatase, MPP1 (also known as p55).

A widely used method for determining clonality using the X chromosome inactivation principle uses a different approach, based on DNA methylation and CAG tandem repeats at the Human Androgen Receptor (HUMARA) locus. The human androgen receptor gene (HUMARA) is located at Xcen-q13 and contains a polymorphic short tandem repeat (STR) in the first exon (Figure 5a; ). The number of tandem CAG repeats are different on the maternal and paternal X chromosomes. The methylation status of the
gene distinguishes inactive and active X chromosomes, because it has been demonstrated that the methylation of HpaII and HhaI restriction sites near the polymorphic STR is correlated with X chromosome inactivation. Therefore, when the DNA isolated from the female is treated with Hpa II or HhaI restriction enzymes, unmethylated (active) alleles of HUMARA gene are digested by the enzyme, while methylated (inactive) ones remain unaffected. Accordingly, after digestion, only methylated alleles are amplified by PCR and can be visualized as products separated by agarose gel electrophoresis (Figure 5b). The HUMARA method is more widely applicable than protein isoform and transcription-based methods, because the variable number of CAG nucleotide repeats makes most individuals and patients informative for the assay.

The chemical modification of cytosine to uracil by sodium bisulfate treatment provides another method for the study of DNA methylation that avoids the use of restriction enzyme. In this reaction, all cytosine residues are converted to uracil by sodium bisulfite, but those that are methylated (5-methylcytosine) are resistant to this modification and remain as cytosine. By taking advantage of the sequence differences resulting from bisulfate modification, primers can be designed to distinguish methylated from unmethylated DNA in bisulfate modified DNA. Modified and unmodified alleles can be amplified by PCR and the products can be visualized as bands by ethidium bromide on agarose gel after electrophoresis. The number of the visible bands for methylated and unmethylated primer pairs determines the clonal pattern of the studied cell population (Figure 5b). Unmodified DNA or DNA incompletely reacted with bisulfate can also be distinguished by this method, since marked sequence differences exist between these DNAs. The frequency of CpG sites in CpG islands renders this technique uniquely useful and extremely sensitive for such regions. This method is named as Methylation Specific PCR for HUMARA gene (HUMARA-MSP).
Figure 5: (a) Schematic representation of the region containing polymorphic CAG repeats in the first exon of the human androgen-receptor (HUMARA) gene. (b) Schematic representation of the expected results of typical samples by the HUMARA assay and those by the HUMARA-MSP assay. ‘A pattern’ cells have a methylated maternal allele, whereas ‘B pattern’ cells have a methylated paternal allele. U, PCR products amplified with primers specific for unmethylated DNA; M, PCR products amplified with primers specific for methylated DNA.

It is important to understand both the initial steps of mesothelioma development and the mechanisms controlling tumor progression as well, to identify possible therapeutic targets and tools. Ion channels, being abundant in all eukaryotic cells and regulating a variety of pathways critical for several cellular functions, including survival, proliferation, migration and apoptosis, may be valid targets to inhibit a variety of intracellular signaling pathways relevant for tumor progression. Recent studies on members of the Melastatin Like Transient Receptor Potential (TRPM) ion channel family associated these ion channels with cancers, such as glioblastoma and prostate carcinoma.
To date no study has been conducted to investigate the relationships between any TRPM ion channel and mesothelioma, therefore investigating such a possible link might reveal novel approaches to reduce the aggressiveness of this cancer.

4.3. TRPM Ion Channels

The organization of cells in a multicellular organism depends on rapid and accurate transmission of information. Electrical signals and chemical compounds induce changes of intracellular second messenger concentrations, e.g., calcium ions, cAMP and cGMP. In excitable cells, electrical signals induce increases in the intracellular calcium concentration, necessary for cellular responses like hormone secretion, contraction of muscle cells and detection, processing and response to environmental stimuli. These functions are mediated by voltage-gated calcium channels and hyperpolarization-activated cyclic nucleotide-modulated channels. The molecular basis for the hormone-induced calcium transient increase in non-excitable cells was unraveled with the molecular characterization of the TRP locus of the Drosophila genome. Montell and Rubin identified and cloned Drosophila TRP as a cation channel involved in Drosophila phototransduction. The sequencing of the different genomes accelerated the access to homologous proteins in worms and humans, and soon the TRP channels grew up to a superfamily (Figure 6;) with more than 20 different genes, coding for TRP-homologous channel proteins in humans. The classification of the 20 TRP channels into at least three subfamilies (TRPC, TRPV, TRPM) was initially based on sequence comparison, later the functional data helped to structure the variety of proteins.
The TRP superfamily of ion channels is classified into three main homologous subfamilies (TRPC, TRPV and TRPM) and more-distantly related groups (TRPML, TRPP, TRPN and TRPA). All TRP channels are characterized by six transmembrane regions with cytoplasmic N- and C-termini. Based on homology, the TRPM subfamily is divided further into four pairs of homologous channels (TRPM1 and TRPM3; TRPM2 and TRPM8; TRPM4 and TRPM5; and TRPM6 and TRPM7).

Proteins closely related to the Drosophila TRP channel protein on the basis of phylogeny and function form the family of classic TRP channels (TRPC, (93)). The TRPV family is formed by proteins related to the vanilloid receptor 1 or capsaicin receptor 1. The TRPM group was named according to the first known member, melastatin, a protein identified in a comparative screening on benign and malignant melanocytes. Within the superfamily of TRP channels, the proteins of the TRPC and TRPV classes are best characterized, whereas there are deficits in understanding the function of the many TRPM members. Comparisons across the species resulted in 1, 3 and 8 TRPM members in Drosophila, C. elegans and mammals, respectively. The human TRPM subfamily consists of eight members that can be grouped into four pairs: TRPM1 and TRPM3; TRPM2 and TRPM8; TRPM4 and TRPM5; and TRPM6 and TRPM7. Most of the TRPM members have been characterized by electrophysiology in heterologous expression systems and native cells. From these studies, it is evident that the TRPM subfamily represents a heterogeneous group of ion channels with diverse selectivity, activation mechanisms, and characteristic kinetics of activation and inactivation (Table 1; ).

<table>
<thead>
<tr>
<th>Channel</th>
<th>Gating</th>
<th>Voltage Dependent</th>
<th>Regulation</th>
<th>Ion Selectivity</th>
<th>Expression</th>
<th>Function</th>
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<td>ND</td>
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<td>Non-selective?</td>
<td>in malignant melanoma</td>
<td>Cell proliferation</td>
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<td>TRPM2</td>
<td>ADP-Ribose, NAD, H2O2</td>
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<td>[Ca2+]i, [Na+]i</td>
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<td>ATP-4</td>
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Table 1: Properties of TRPM* Ion Channels.
4.3.1. TRPM1

In a differential display screening of different melanoma cell lines, the mRNA of TRPM1 was identified and named melastatin, because of the absence of TRPM1 mRNA in malignant transformed melanoma cell lines, suggesting a tumor suppressor function of the channel protein. The supposed role of TRPM1 in cellular differentiation and proliferation processes is further confirmed by studies of hexamethylene bisacetamide (HMBA)-treated human pigmented melanoma cells. HMBA-treated cells differentiate and change pattern of expressed proteins. Pigmented metastatic melanocytes selectively transcribe TRPM1 mRNA. The level of TRPM1 mRNA could be enhanced by incubation in the presence of 5 mM HMBA. Furthermore, the existence of a large number of splice variants was described. Analysis of signals from Northern hybridization studies revealed the presence of at least four different transcripts coding for TRPM1 proteins (1.3, 1.8, 4.5, 5.4 kb mRNA). The transcription of the individual mRNA species depends on melanoma cell lines and differentiation states. The available Expressed Sequence Tag-based (EST) profiling of human embryonic stem cells (ESC) indicates the presence of TRPM1 mRNA during all stages of embryonal development, whereas in tissues of adults TRPM1 mRNA is restricted to skin and eye. Although melastatin is the first member of the TRP family, little is known about its functional properties and cellular functions.

4.3.2. TRPM2

TRPM2 is a channel-enzyme or “chanzyme” within the TRP family, forming a calcium-permeable cation channel, fused to an enzymatic domain with ADP-ribose pyrophosphatase activity at the cytosolic C-termini of the protein. Its full length molecular weight was determined as 170 kDa. It has been proposed as a regulator of a number of signaling cascades that have physiological roles, such as cell death, cytokine production and insulin secretion. TRPM2 cleaves ADP-ribose, a breakdown product of NAD and cyclic ADP-ribose, representing an intracellular second messenger stimulating calcium release, mediated by ryanodine receptors. The activity of TRPM2 depends on the presence of intracellular calcium and is induced by extracellular application of hydrogen peroxide (Figure 7). The activation of TRPM2 by hydrogen peroxide is probably linked to the activity of the poly(ADP-ribose) polymerase, an enzyme transferring multiple ADP-ribose groups to proteins. Oxidative stress and other stimuli causing DNA damage enhance poly(ADP-ribose) polymerase activity. The large and branched structure of the poly(ADP-ribose) modifications are reduced prior to protein degradation to mono(ADP-ribose) by the poly...
(ADP-ribose) glycohydrolase, a process releasing ADP-ribose. Evidence for this intracellular pathway resulting in TRPM2 activation has been confirmed by the use of inhibitors of poly(ADP-ribose) polymerase, which were able to interfere with the hydrogen peroxide-induced TRPM2 activation. Whereas TRPM2 is insensitive to lanthanum and gadolinium ions, it was recently shown that TRPM2 currents are blocked by flufenamic acid, a compound known to block a great variety of channel proteins. Flufenamic acid has been characterized as an open-channel blocker of TRPM2. Activity of flufenamic acid depends on the pH with enhanced effect at acidic conditions.

TRPM2 expression was detected in pancreas and cell lines derived from pancreatic islet cells. In brain, TRPM2 is mainly expressed in the immune cells of the brain, the microglia and its sensitivity to hydrogen peroxide depends on the activation state of the microglia. This suggests that TRPM2 is involved in the regulation of intracellular calcium depending on the developmental state of the microglia. In summary, TRPM2 is a hydrogen peroxide-activated cation channel involved in the host-defense system of the body.

4.3.3. TRPM3

The transcription of the TRPM3 gene results in a vast number of different mRNA species. In Northern Blot analyses of mouse brain at least three transcripts of different lengths were detectable, whereas Lee et al. cloned six variants from human kidney, which vary in short deletions and insertions indistinguishable by Northern Blot analysis. TRPM3 protein has been shown to be expressed in human brain and human kidney, while in mouse kidney it is undetectable. The different lengths of TRPM3 mRNAs and the apparent molecular masses of the TRPM3 proteins probably result in different activation mechanism. The
long variant encoded by 1545 amino acids is probably activated by store-depletion, the short variant encoded by 1325 amino acids; however, forms a channel protein of approximately 150 kDa which mediates calcium entry upon extracellular application of hypotonic solution. The TRPM3-mediated hypotonicity induced calcium entry can be blocked by the nonselective blockers of cation channels, such as lanthanum and gadolinium ions. The expression of TRPM3 in kidney and the activation by hypotonicity argue for the function of TRPM3 in renal osmo-homeostasis.

4.3.4. TRPM4

TRPM4 is a calcium-activated sodium channel that is impermeable for calcium and it mediates the depolarization of the plasma membrane. TRPM4 has been described to occur ubiquitously. Its down-regulation in cerebral vascular smooth muscle cells results in attenuated myogenic constriction, arguing for the regulation of cerebral blood flow by TRPM4-dependent plasma membrane depolarization. TRPM4 is activated by intracellular calcium, modulated by voltage and inhibited by ATP, ADP, AMP and intracellular application of polyamines like spermine.

4.3.5. TRPM5

Like TRPM4, TRPM5 also forms calcium-activated sodium channels impermeable for calcium and mediate depolarization of the plasma membrane. It is selectively expressed in stomach, intestine and cells of the taste buds, suggesting an involvement in taste transduction. TRPM5 has a transient nature with fast activation and inactivation kinetics and it responds to Ca\(^{2+}\) release from intracellular stores. These features make TRPM5 suited to coupling agonist-mediated Ca\(^{2+}\) release from intracellular stores with electrical activity.

4.3.6. TRPM6

TRPM6 is selectively expressed in intestinal and renal epithelia and involved in intestinal uptake and renal re-absorption of magnesium. Mutations in the TRPM6 gene are linked to the hereditary disease of familial hypomagnesemia with secondary hypocalcemia. The mutations found in patients often result in truncated proteins or in variants with deficits in the translocation process to the plasma membrane. Heterologous expression of TRPM6 forms a magnesium- and calcium-permeable cation channel. This cation channel can be regulated by magnesium and blocked by ruthenium red in a voltage-dependent manner.

4.3.7. TRPM7
The ubiquitously expressed TRPM7 was involved in the regulation of cellular magnesium homeostasis and in the regulation by G-protein-coupled receptors, upon hormone stimuli (GPCR). The molecular weight of full length TRPM7 protein was determined as 140 kDa. The group of David Clapham characterized TRPM7 as a phospholipase C-interacting TRP channel (TRP-PLIK) and provided evidence for an activation mechanism dependent on the phosphoinositol-4,5-bisphosphate (PIP2) concentration and phospholipase C activity (Figure 8). Their data suggested that TRPM7 is activated by a reduced PIP2 concentration resulting from breakdown of phosphoinositides by phospholipase C and inhibited by the activity of phosphoinositide kinases restoring the plasma membrane PIP2 concentration. The group of Andrea Fleig and Reinhold Penner proposed an alternative activation pathway in which cAMP-dependent phosphorylation of TRPM7 is induced by GPCR (Figure 8). Their experiments showed differential modulation of TRPM7 activity after β-adrenergic stimulation or muscarinic activation of the cells. Whereas β-adrenergic resulted in increased TRPM7 activity, muscarinic stimulation inhibited TRPM7 currents in a pertussis toxin-dependent manner. Further characterization revealed that the TRPM7 activity depends on the intracellular cAMP concentration and a functional cAMP-dependent protein kinase. The variety of proposed activation mechanisms was enhanced by the description of the involvement of TRPM7 in neurotoxic death. Cellular calcium overload mediated by N-Methyl-D-aspartic acid (NMDA) receptors in brain ischemia is thought to be a major trigger causing neuronal cell death. However, the pathogenesis is very complex and additionally triggered by increased concentrations of reactive oxygen species (ROS) during the reperfusion phase. In cultured neurons, the down-regulation of TRPM7 by siRNA was associated with reduced anoxic cell death and decreased ROS production, arguing for a model in which a triggering increased intracellular calcium concentration is mediated by NMDA receptors during ischemia and by TRPM7 during the reperfusion phase. It is unclear whether TRPM7 is modulated by the different activation mechanism synergistically or independently. In summary, TRPM7 is ubiquitously expressed and it is involved in the regulation of cellular magnesium homeostasis.
4.3.8. TRPM8

The cDNA of TRPM8 was isolated from prostate cancer cells, and the function of TRPM8 was initially linked to progression of cancer cells. The physiological role of TRPM8 as a sensor of low temperature, or "cold receptor", of the body was revealed by an expression cloning approach to identify a menthol receptor from trigeminal neurons. The isolated cDNA codes for TRPM8 and forms a calcium-permeable cation channel. In TRPM8-expressing cells, application of menthol, icilin or other cooling agents induce TRPM8 currents, which are comparable to activation of TRPM8 by temperatures lower than 28°C. Furthermore, TRPM8 is activated by many other odorant agents isolated from plants, e.g., linalool, geraniol, and hydroxycitronellal. TRPM8 expression is detected in prostate and other tissues of the urogenital tract, but high TRPM8 expression is specifically found in a subset of pain- and temperature-sensing neurons.
5. RATIONALE AND HYPOTHESIS:

According to the histological classification, MM can present with epithelial morphology (epithelioid), a fibrous morphology (sarcomatoid), or a combination of both (biphasic). Most MMs (approximately 50-60%) are of the epithelioid type, approximately 10% are sarcomatoid, and the remainders are biphasic. Moreover, rare MM cannot be histologically classified and are indicated a poorly differentiated malignant mesothelioma. The histological classification is relevant for an accurate prognosis, because MM with prevalent sarcomatoid morphology are chemo-resistant and patient median survival does not reach more than one year from diagnosis, while epithelioid MM are associated with prolonged survival up to two years from diagnosis. In general, at least 10% of the tumor must have a fibrous (or epithelial) component for the malignant mesothelioma to be classified as biphasic. However, even a very tiny fraction of the tumor cells show both morphologies. This unusual variability in morphology of the tumor cells might be an indication for the multiple originators of the mesothelioma tumor.

Ion channels play a crucial role in a variety of physiological functions such as excitability, muscle contraction and hormone secretion. In addition of these life supporting activities, ion channels are also associated with several diseases, including cancer. Specifically, the importance of TRPM2 and TRPM7 Ion Channels in cell proliferation has recently been proven in various tumor cells. Consistently with these results, our preliminary studies showed a time- and dose-dependent relationship between asbestos exposure and the increase in TRPM2/TRPM7 expressions.

On the basis of these results, we hypothesized that A) Malignant mesothelioma tumor cells might have a polyclonal origin, as a characteristic feature of this malignancy. B) TRPM2 and TRPM7 ion channels may be required for primary mesothelial cells to survive asbestos exposure and to undergo transformation. C) TRPM2 and TRPM7 ion channels might have important roles in MM tumor progression. D) Down regulation of TRPM2 and TRPM7 genes in MM cells may affect their tumorigenic properties, such as rapid growth, wound healing and migration.

5.1. AIMS

Our study aimed at investigating: 1) the clonal origin of MM tumor cells and 2) the tumorigenic potential of TRPM2 and TRPM7 ion channels in MM.

Our first goal was to determine the clonal origin of MM tumors. We used X chromosome methylation based techniques to approach this goal, and therefore, only tumors from female patients could be used.

The second goal of the study was to investigate a possible correlation between TRPM2 and TRPM7 ion channels and malignant mesothelioma. We investigated the correlation in two different time points of
tumorigenesis. First, we explored the effect of asbestos on TRPM2 and TRPM7 ion channel mRNAs by exposing immortalized mesothelial cells to low (5µg/cm²) and high (10µg/cm²) amounts of crocidolite in a panel of different time points (from 6 hours to 1 week). Second, we investigated the role of the ion channels in transformed cells (MM cell lines) by silencing the expression of their genes.

5.1.1. Aim 1

To determine whether mesothelioma tumors are monoclonal or polyclonal. To investigate the clonal origin for mesothelioma tumors of female patients.

5.1.2. Aim 2

To elucidate the role of TRPM2 and TRPM7 ion channels in asbestos induced cell transformation.

5.1.2.1. Aim 2A

To evaluate the levels of expression of TRPM2 and TRPM7 ion channels in mesothelial cells and MM cells.

5.1.2.2. Aim 2B

To evaluate the expression and the activity of TRPM2 and TRPM7 ion channels in mesothelial cells exposed to asbestos crocidolite fibers.

5.1.3. Aim 3

To elucidate the role of TRPM 2 and TRPM7 ion channels in MM. To investigate how silencing of TRPM2 and TRPM7 genes affects proliferation, migration, and invasiveness of MM.

5.1.4. Aim 4

To determine the exact localization of TRPM2 ion channels in HM and MM.

6. INNOVATION AND IMPACT

Detection of the clonal pattern in MM can contribute to understand the carcinogenesis mechanisms of this cancer, with translational implications for both diagnosis and prognosis. Clonal origin information provides the experimental evidence to have a more realistic tumor initiation hypothesis for a given tumor.
Additionally, such knowledge could be useful to develop better strategies to eliminate the tumor since a polyclonal tumor could be considered as a heterogenous population of cells and therefore its elimination might require a combination of different treatments.

Up to date, at the best of my knowledge, no study has evaluated the clonal pattern of MM or the role of TRPM2 and TRPM7 Ion Channel Activities in asbestos-induced mesothelioma. Therefore, all the data obtained through this thesis will be novel. Discovering the clonal pattern of mesothelioma tumors will provide us with a better understanding of the natural process of tumor initiation in this malignancy while the evaluation of the potential functions of TRPM2 and TRPM7 in MM will lead further knowledge on the molecular biology of HM and MM. Moreover, by exploring the possible role of these ion channels in cell proliferation, migration or apoptosis in MM may reveal novel targets for treatment or prevention of this cancer.

7. RESULTS

7.1. Aim 1

To determine whether mesothelioma tumors are monoclonal or polyclonal.

7.1.1. Rationale

In a patient of MM the tumor usually presents with two or more anatomically distinct and histologically similar pre-malignant lesions. Whether these multiple early lesions arise from the same initial dysplastic event is still not known. It is generally accepted that the development of a neoplasm is a multistage progressive process. During the process of cellular transformation and development of a neoplasm, multiple genetic events are known to accumulate in the neoplastic cells. However, it is unlikely that multiple events occur in a single cell. It is the clonal nature of neoplastic growth that enables rare events to accumulate in the cell population of a tumor. A clonality assay, applied to small portion of tumor tissue, after removal of non-neoplastic tissue, should demonstrate the clonal origin of solid tumors. With this approach, we would like to address the issue of whether a macroscopic lesion in MM is derived from a single cell or a clone (monoclonal carcinogenesis) or multiple clones are involved in the formation of the lesion (field cancerization). No attempts have been made to target this issue in mesothelioma so far. In our study, we aim at determining the clonal origin of MM tumor cells by using two widely validated methods; HUMARA and HUMARA-MSP.

7.1.2. Method optimization

HUMARA and HUMARA-MSP are two widely accepted assays used to determine the clonal origin of a tumor cell population. They both exploit a natural event that occurs during female embryonic development. This event, called X chromosome inactivation, provides a practical tool to track the progenitor of a cell population thanks to the fact that the same X chromosome remains inactive
(methylated) through the generations of the cells derived from the same founder. By using the methylation status of Human Androgen Receptor Gene (HUMARA) that is encoded in X chromosome, it is possible to differentiate a monoclonal population from a polyclonal one. A monoclonal population presents a single type methylated X chromosome in its cells while two types of methylated X chromosomes are observed in a polyclonal population. Although the two techniques take advantage of the same natural event, the way they discriminate the methylated allele from the unmethylated is quite different. HUMARA assay uses a restriction enzyme (HpaII) that can digest the unmethylated DNA, thereby resulting in the amplification of methylated DNA only. HUMARA-MSP, on the other hand, uses a chemical solution (Sodium Bisulfite, NaHSO₄) that causes the conversion of Cytosine bases into Uracils in the unmethylated DNA. Thus, the regions that will be amplified become different in the methylated and the unmethylated alleles and they can be amplified by different sets of primers. Discrimination of monoclonal and polyclonal tumors through HUMARA assay can be made by considering the number of the PCR products that range between 200 and 250 bp. Single size PCR product for the HpaII digested tumor DNA represents one methylated allele in the population and refers to a monoclonal origin for the tumor. PCR products with two different sizes; however, indicate a polyclonal origin for the tumor. In HUMARA-MSP assay, two sets of PCR products can be generated. One set is amplified by methylated DNA specific primer, the other is amplified by unmethylated DNA specific primer. If the amplification of converted tumor DNA by each of these primer sets produces a single size PCR product, then tumor is thought to be derived from a single cell or clone (i.e., monoclonal). However, if two primer sets produce two differently sized PCR products, then tumor is considered to have multiple progenitors (i.e., polyclonal).

We applied both techniques to our samples and we obtained comparable results. However, the sensitivity of HUMARA-MSP was generally lower than HUMARA, possibly due to the degradation of DNA during the bisulfate treatment. Furthermore, a higher amount of DNA was needed for HUMARA-MSP assay, and obtaining the required amount of DNA was a challenge, because of the paucity of available material and because the dissection of tumor tissues from slides is a very time consuming process. Although we decided to perform preferentially HUMARA assay on most of the samples, semi-positive HUMARA-MSP results for three tumor samples are represented in Figure 9. These results were later confirmed by HUMARA assay.
HUMARA-MSP assay results for three tumor samples. HUMARA-MSP assay was applied to DNA extracted from mesothelioma of three female patients, labeled as 61, 93, and 207. M and U: primers amplifying methylated and unmethylated alleles of HUMARA gene, respectively. The amplification products either of the methylated or of the unmethylated alleles should be resolved in two bands to define the polyclonal origin of the sample; however, if two bands are present only in one of the products this also suggests a polyclonal origin for the sample studied.

To ensure that HUMARA assay can be used to distinguish the clonal pattern of a cell population with variable ratios of monoclonal and polyclonal origins, we performed a titration experiment in which DNA mixtures of different amounts of monoclonal and polyclonal controls were amplified with PCR. This technique was able to identify a sample, mimicking the polyclonal pattern, when less than 50% of the mixture was monoclonal (Figure 10). DNA samples that were amplified by HUMARA assay were also genotyped with Applied Biosystems 3130xl Genetic Analyzer (see below).
Figure 10: HUMARA assay for different ratios of monoclonal and polyclonal DNA mixtures. In order to establish the sensitivity of the HUMARA assay for detecting minor alleles, different amounts of HpaII digested (mono-allelic) and non-digested (bi-allelic) DNA from cell line 1290 (monoclonal melanoma) were mixed. The minor allele was visible in PCR reactions that incorporated as little as 25% of the bi-allelic sample. Two bands on the gel indicate a polyclonal origin for the cell population from which the DNA was extracted, while one band proves the monoclonality. N stands for Negative Control. 100, 75, 50, 25 and 0 indicate the percentage of bi-allelic DNA in the sample loaded.

7.1.3. Preparation of DNA samples

Tumor samples from women who underwent surgery for the treatment of MM were obtained from Dr. H. I. Pass (NYU, New York) and from Dr. Paul Sugarbaker (WCI, Washington, DC) in accordance with protocols approved by the Institutional Review Board of each center and upon patients informed consent. Early stage lesions were selected for this study and the identification of tumor and normal tissues in each sample was performed by Hematoxylin-Eosin (H&E) staining. Tumor and Normal cells were dissected from the slides by Laser-Capture Microdissection (LCM) separately (Figure 11). LCM tubes were incubated for 48 hours at 37°C, centrifuged, and subjected to protein digestion for two additional days at 55°C, by adding fresh Proteinase K daily. DNA was extracted by using DNeasy Blood&Tissue Kit (Qiagen, Valencine, CA). Slides were prepared from frozen tissues, and stained freshly with H&E before microdissection.

Figure 11: A representative tumor section is shown before and after tumor cells were collected by microdissection. Tumor cells from tumor sample #93 were collected using Laser Capture Microdissection.

7.1.4. HUMARA Assay

A fraction of 250 ng of each DNA extracted separately from tumor or normal cells was digested with 10U of Hpa II restriction enzyme (New England BioLabs) overnight at 37°C. Another fraction of 250 ng was
subjected to mock digestion in absence of the enzyme. After incubation, enzyme activity was inactivated at 65°C for 10 minutes. Subsequently, DNA was purified by using Wizard DNA Clean Up System (Promega, Madison, WI) and stored in 20μL aliquots of TE Buffer. Then HUMARA assay was performed as described in the Materials and Methods section. PCR products of every experiment were electrophoresed on 3% Agorose Gel and stained with 0.05% Ethidium Bromide to visualize bands under Ultraviolet (UV) light. In order to ensure that the results generated through this technique would represent the true clonal nature of the tumors, we ran three sets of control DNA in each experiment along with the sample tumor studied. Figure 12 is a representative gel picture of our controls processed through HUMARA assay. Mock digested Marf 11 (-), as the undigested DNA extracted from a male, showed a single band indicating the presence of a single X chromosome. When digested (Marf11 +), the sample presented no visible bands on the gel, reflecting the efficiency of our digestion to eliminate the unmethylated DNA. Mock digested DNA from a female melanoma cell line (1290-) presented two bands, as expected from a female cell population. Overnight digestion of this sample with Hpall (1290+) produced a single size PCR product, which proved both the elimination of unmethylated X chromosome by enzyme digestion and the protection of methylated X chromosome against the digestion. As a positive polyclonal control, DNA extracted from a healthy female (L-IV-11) was used. Presence of two bands for mock digested (L-IV-11-) and digested (L-IV-11+) sample signified the efficiency of our technique to demonstrate the true polyclonal nature of a polyclonal cell population. In Figure 13, a polyclonal pattern was evident from the occurrence of two bands in both mock-digested and digested samples for four representative MM samples (#61, #93, #207 and #R693). All informative MM samples analyzed in this study revealed the same pattern.

**Figure 12: Controls for HUMARA Assay**: PCR products for mock digested (-) and Hpall digested (+) control DNA were electrophoresed through 3% Agarose gel and visualized using Ethidium Bromide.
Marf11, DNA extracted from a male's blood, was used to check the efficiency of enzyme (HpaII) digestion. One band for the mock digested sample confirmed that it was a male DNA, and no visible band for the digested sample proved that the enzyme conditions were sufficient to completely digest the unmethylated DNA. 1290, a melanoma cell line, was the positive monoclonal control. Two visible bands in the mock digested lane confirmed that it was a female cell line, and the disappearance of one of the bands in the digested lane represented the efficiency of the technique in visualizing the monoclonal pattern of a cell population. L-III-4, DNA extracted from a healthy female, was used as the positive polyclonal control. The presence of two bands for both mock digested and digested samples was the evidence that polyclonal pattern of a cell population can be observed by HUMARA assay.

![Image of gel electrophoresis results](image)

**Figure 13:** The HUMARA assay demonstrates mesotheliomas are polyclonal in origin. PCR products from mock digested (-) and HpaII-digested (+) samples were electrophoresed through a 3% agarose gel and visualized using ethidium bromide. In each panel, 'Normal' refers to the DNA extracted from non-tumoral (stromal) cells in the tumor micro-environment while 'Tumor' refers to the DNA extracted from tumor cells. Presence of two bands in HpaII digested tumor samples proves that two different methylated X chromosomes exist in the tumor cell population, which indicates a polyclonal origin for the tumors.

An aliquot of 10 µl of each PCR product was genotyped in the University of Hawaii Biotechnology facility (Honolulu, HI) with the Applied Biosystems 3130xl Genetic Analyzer. Results were presented as fluorescent peak trace chromatograms. The composite nature of each peak is due to the intrinsic inaccuracy of the polymerase mediated amplification (Figure 14). The analysis performed on digestion control (Marf11; male DNA), monoclonal positive control (1290; female melanoma cell line) and polyclonal positive control (L-IV-11; healthy female DNA) validated the accuracy and specificity of the technique. In the digested digestion control (Marf11+), the disappearance of the single peak that was present in the mock digested sample (Marf11-) further validated the efficiency of our digestion conditions. Positive monoclonal control (1290) lost one of its peaks products when digested (1290+), proving that a monoclonal population with one methylated allele of HUMARA gene could be determined by our method. Positive polyclonal control (L-IV-11) presented two peaks for both mock digested (-) and digested (+) samples. Considering the fact that our digestion conditions are efficient and the number of the methylated
alleles can be determined by the number of the peaks in the expected size range (200-250bp), it was evident that polyclonal pattern of a cell population can sufficiently be detected by this assay. Although the peaks roughly revealed the same information as it was in gel pictures, knowing the exact sizes of the PCR products was a helpful tool for generating data for the samples that produced poorly visible bands on the gel or for understanding the nature of the uninformative samples (See: 6.1.5. A Peculiar Case).

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<th>PCR Product Size (bp)</th>
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**Figure 14**: Genotyping images of the controls used for HUMARA assay. PCR products of mock digested (-) and digested (+) samples obtained from HUMARA assay were genotyped. Fluorescent peak trace chromatograms for each sample are presented here. DNA extracted from a male's blood (Marf11) was used to check the enzyme digestion efficiency. One peak for the mock digested sample (Marf11-) confirmed that it was a male DNA, and no peak for the digested sample (Marf11+) proved that the enzyme conditions were sufficient to completely digest the unmethylated DNA. DNA from the melanoma cell line 1290 was used as the monoclonal control. Two visible peaks in the mock digested (1290-) sample confirmed that it was a female cell line, and the disappearance of one of the peaks when the sample was digested (1290+) reflected the efficiency of the technique in visualizing the monoclonal pattern of a cell population. L-IV-11, DNA extracted from the blood of a healthy female, was used as the polyclonal control. The presence of two peaks for both mock digested (L-IV-11-) and digested samples (L-IV-11+) was the evidence that polyclonal pattern of a cell population can be observed by HUMARA assay. The numbers on the upper line reflect the length of the PCR products in terms of base pairs. The surface areas of the peaks are proportional to the quantity of PCR products.

The genotyping of the three representative MM samples (#61, #93, #R693) was conducted both on the normal tumor-surrounding tissue and on the corresponding tumor tissue, isolated by LCM. The results obtained on the PCR products from non tumor cells (normal surrounding tissue) showed a clear polyclonal pattern, as expected (Figure 15). The genotyping results of the three representative MM samples confirmed that also the actual pattern of tumor cells was polyclonal (Figure 16). The differences in molecular size among the different samples are due to the variability of the numbers of CAG repeats.
Figure 15: Genotyping images of the three tumor surrounding normal cells. HUMARA assay was performed to the normal cell DNA surrounding the tumor and the PCR products were genotyped. Two peaks located between 200-250 bp confirmed the polyclonal origin of the samples. The numbers on the upper line reflect the length of PCR products in terms of base pairs. The surface areas of the peaks are proportional to the amounts of PCR products in each reaction.
Genotyping patterns of three tumor samples. PCR products of three tumor samples (#93, #61 and #R693) obtained from HUMARA assay were genotyped and two peaks ranging 200-250 bp were observed for each sample, suggesting a polyclonal origin for the tumors. Numbers on the upper line show the length of the PCR products in terms of base pairs. The surface areas of the peaks are proportional to the quantity of the PCR products in each reaction.

Even though HUMARA assay was more reliable in determining the clonal patterns of the tumors comparing to HUMARA-MSP assay, this technique had its own limitations as well. The reaction of enzymatic digestion with HpaII had negative consequences on the DNA purity, both because of the reagents added and of the star activity of this enzyme. Thus, a further step of purification was needed subsequent to digestion and this may cause DNA loss. Therefore, the concentration of the PCR product was sometimes inadequate to generate visible bands on Agorose Gel. In order to overcome this problem, we decided to perform a Semi-Nested PCR in which a primer pair that is able amplify a shortened version of the target region is designed. An aliquot of 1 µl from the conventional PCR products (diluted 1:100) was used as template in Semi-Nested PCR. Figure 17 shows the pattern of Semi-Nested PCR products.
of DNA from normal and tumor cells of the representative tumor sample #273. We were unable to visualize their products by conventional PCR (data not shown).

Figure 17: Semi-Nested PCR products of mock digested (-) and digested (+) DNAs extracted from normal and tumor cells of Tumor-273. The occurrence of two bands both in undigested (-) and digested (+) samples reveals the polyclonal origin of both normal and tumor cell populations in the sample.

These data indicate a polyclonal origin for the MM tumors analyzed.

7.1.5. A peculiar case

In a set of experiments, we studied the tumor sample L-III-18 from a panel of familial mesotheliomas, aiming at determining its clonal pattern. When HUMARA-MSP assay was applied to the DNA extracted from normal surrounding and tumor cells of this specimen, an unusual pattern of bands was observed (Figure 18). The electrophoretic separation of both normal and tumor cell DNA revealed only one band for both the methylated and the unmethylated alleles. Initially, we considered this sample as an example of Skewed X Chromosome, an event that is seen in nearly 10% of the population. Skewed X chromosome Inactivation represents a non random selection of X chromosome to be activated. Therefore, instead of having 50% methylation for each allele, people with this condition show a 100 % methylation for one allele and none for the other. However, a further investigation of the sample by amplifying the gene without enzyme digestion in HUMARA assay also produced a single band on the gel (data not shown). Since there was no digestion before amplification, two alleles were amplified regardless their methylation status. In this case, seeing one band could only mean that, in this patient, the two alleles of the gene we work with were at the exact size if she does not have an X0 genotype. Since we saw single bands for both the
methylated and the unmethylated alleles in HUMARA-MSP, the possibility of this individual having X0 genotype has been eliminated (In case of X0, there would be only a band for the unmethylated allele because the methylation of the only existing X chromosome would be fatal for the cells). Moreover, the similarity of the electrophoretic mobility between the amplification products of the two alleles with methylated and unmethylated primers also suggested the presence of two identical alleles of HUMARA gene in this specimen.

Figure 18: HUMARA-MSP results of sample L-III-18. DNA obtained from normal and tumor cells of the tumor sample was treated with Sodium Bisulfite and amplified with methylated (M) and unmethylated (U) allele specific primers. The identical size of methylated and unmethylated primer products suggests the presence of two identical alleles of HUMARA gene in this individual.

Furthermore, when the PCR products obtained from HUMARA assay performed on the normal and tumor cell DNA of L-III-18 were genotyped by the University of Hawai‘i Biotechnology Facility, the results showed only one peak, in the 200bp-250bp range both in normal and tumor DNA after digestion (Figure 19). The peaks were coincident in the two samples and indicated the presence of only one type of methylated X chromosome.
Figure 19: Genotyping results of sample L-III-18. HUMARA assay was performed on normal and tumor cell DNA obtained from the sample and the PCR products were genotyped. One peak in the 200bp-250bp range was observed for both normal and tumor cell DNA, confirming the fact that X chromosomes of this individual are the exact same size, therefore it is not informative for our purpose.

Although the result from this sample was defined as non informative for clonal origin determination, it further validated the accuracy of our methodology.

7.2. Aim 2

The transient potential receptor melastatin-2 (TRPM2) channel has emerged as an important Ca$^{2+}$ signaling mechanism in a variety of cells, contributing to cellular functions that include cytokine production, insulin release, cell motility, and cell death. Its ability to respond to reactive oxygen species has made TRPM2 a potential therapeutic target for chronic inflammation, neurodegenerative diseases, and oxidative stress-related pathologies.
TRPM7 is endogenously expressed in a wide variety of tissues including brain and hematopoietic tissues as well as kidney and heart tissues. The TRPM7 cation channel supports multiple cellular and physiological functions, including cellular Mg\(^{2+}\) homeostasis, cell viability and growth, anoxic neuronal cell death, synaptic transmission, cell adhesion, and intestinal pacemaking. TRPM7 channel is critical for human mast cell survival and plays an important role in human osteoblast-like cell proliferation.

It is well known that long term asbestos exposure results in transformation of mesothelial cells into mesothelioma; however, thus far, the possible functions of TRPM2 and TRPM7 in asbestos induced mesothelial cell transformation have not been investigated.

7.2.1. Aim 2A

To evaluate the levels of expression of TRPM2 and TRPM7 ion channels in mesothelial cells and MM cells.

7.2.1.1. Rationale

TRPM2 and TRPM7 ion channels are expressed in nearly all eukaryotic cells including primary human mesothelial cells. Both channels are involved in the transport across the membrane of ions playing important roles in cell homeostasis, because they can be cofactors of important enzymes (e.g., Mg\(^{2+}\) for kinases) or can be directly involved in cellular signaling (e.g., Ca\(^{2+}\) as a secondary messenger). Given the fact that MM cells are originated from primary human mesothelial cells, investigating the different expression levels of TRPM2 and TRPM7 in MM, compared to HM, may reveal a role for those ion channels in this malignancy.

7.2.1.2. TRPM2 and TRPM7 Ion Channel Protein Expression in HM and MM

Primary Human Mesothelial Cells (HM) and Malignant Mesothelioma cells (MM) were analyzed for the expression of TRPM2 and TRPM7 Ion Channels by Western Blotting. TRPM2 protein (170 kDa) was present in every cell type studied (Figure 20a). TRPM2 expression levels may considerably vary among different cell lines. In particular, the fact that both the highest and the lowest expression levels of this protein were observed in MM cells, we concluded that there is no correlation between malignancy of mesothelial cells and TRPM2 protein expression. TRPM7 expression, on the other hand, showed a clear correlation with cell trasformation. All MM cell lines tested (n=7) show expression of the TRPM7 protein, recognizable as a band of 140Kda, whereas all primary HM cells (n=4) were negative (Figure 20b). In view of the fact that TRPM7 has been shown to have a role in cell proliferation in different cell types, we concluded that the difference in TRPM7 levels in HM and MM could reflect the higher proliferation rate of MM cells.
Figure 20: Expression of TRPM2 and TRPM7 in Primary Mesothelial cells and Malignant Mesothelioma cells. Western Blotting analysis of a panel of HM and MM cell lines. A total of 25 µg protein from cell extracts was resolved by electrophoresis on a 8% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were probed with TRPM2 rabbit polyclonal, TRPM7 goat polyclonal, β-Actin (loading control) monoclonal or GAPDH (loading control) monoclonal antibodies, followed by horseradish peroxidase-labelled anti-rabbit, anti-goat and anti-mouse antibodies. The signal was detected by Enhanced Chemiluminescence (ECL). 

a) TRPM2 protein was detected in all HM and MM, whereas it was absent in two out of three immortalized cells (Met5A and LP9).

b) TRPM7 protein was present in all MM but not in HM, suggesting a cell type dependent expression pattern for the ion channel. Transfected human HEK cells expressing high levels of recombinant TRPM2 or TRPM7 were used as positive controls; HM137, HM245 HM246, HM250, HM251, HM258, HM288, HM301, HM315 and HM319, are human primary mesothelial cultures derived from different individuals.
7.2.2. Aim 2B

Determine the expression and the activity of TRPM2 and TRPM7 ion channels in mesothelial cells exposed to asbestos crocidolite fibers.

7.2.2.1. Rationale

Long term exposure to asbestos is one of the main causes of malignant mesothelioma. Altered expression and activity levels of TRPM2 and TRPM7 in human mesothelial cells exposed to asbestos might suggest a role in the asbestos induced malignant mesothelioma.

7.2.2.2. Expression levels of TRPM2 and TRPM7 mRNA

Three different HM primary cultures (HM269, HM270, HM271) were exposed to two different amounts of crocidolite asbestos (5µg/cm\(^2\) and 10 µg/cm\(^2\)) for 6, 12 or 24 hours. At the end of the treatment, mRNA was extracted (RNeasy Kit, Qiagen, CV) and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was performed. Student t was applied to the results to evaluate the differences in TRPM2 and TRPM7 mRNA levels between treated and untreated (0) cells. Differences were considered as statistically significant when p values were smaller than 0.05.

The results revealed a remarkable degree of variability of TRPM2 mRNA levels in the three HM upon asbestos exposure and no association with the amount of fibers or with time of exposure was observed (Figure 21a). TRPM7 expression levels, on the other hand, increased with time when the cells were exposed to the higher amount (10ug/cm\(^2\)) of asbestos, whereas the changes in TRPM7 expression was less consistent among the different HM cultures exposed to 5 µg/cm\(^2\) asbestos. (Figure 21b).
Figure 21: TRPM2 and TRPM7 expression levels in HM cells upon asbestos exposure. Three HM cell lines were exposed to two different amounts of asbestos (5 µg/cm² and 10 µg/cm²) for three different time points (6, 12, and 24 hours). For each cell line, untreated cells were used as controls (0 hours). Extracted mRNA was assayed by qRT-PCR with TRPM2 and TRPM7 specific primers. For normalization purposes, the housekeeper transcript of β-Actin was also amplified. a) The patterns of relative TRPM2 expression levels in three different HM cell lines are shown separately. b) Relative TRPM7 levels in three asbestos exposed HM cell lines are presented individually. n=3, *p<0.05.

The observed high degree of variability could be attributed to the fact that HM were isolated from pleural fluids of different individuals. Therefore, to overcome this individual variability, in the following experiments we used an established cell line from HM immortalized by overexpression of the hTERT telomerase subunit (LP9). We tested two different clones of LP9 from different sources (LP9-Clone A; from Dr. H.I. Pass, NYU and LP9-Clone B; from Dr. J.R. Testa, FCCC) by exposure to the two different amounts of crocidolite (5µg/cm² and 10µg/cm²) for 4 different time points (12, 24, 48, 72 hours). Student t test was applied to the results to evaluate the differences in TRPM2 or TRPM7 mRNA levels between treated and untreated (0) cells. Differences were considered statistically significant when p values were smaller than 0.05. The longer exposure time, compared to the previous experiments, was allowed by the higher survival of these immortalized cells. Expression of TRPM2 transcript in one clone (LP9-Clone A) revealed time- and dose- dependent changes upon the asbestos exposure, whereas the pattern of TRPM2 mRNA expression of the other clone (LP9-Clone B), following asbestos exposure, was irregular (Figure 22a). Similarly, expression of TRPM7 transcript was time- and dose- dependent for the low and high amounts of asbestos in LP9-clone A while the high amount of asbestos resulted in an irregular pattern in LP9-clone B despite the fact time- and dose- dependency of expression of TRPM7 transcript was the highest in this clone when the cells were exposed to the low amount of asbestos (Figure 22b).
Figure 22: Relative TRPM2 and TRPM7 expressions in two immortalized HM Lines exposed to asbestos. LP9 immortalized primary mesothelial cells from two different sources were exposed to two different amounts of asbestos (5 µg/cm² and 10 µg/cm²) for 4 time points (12, 24, 48, 72 hours). Non-exposed cells were used as controls (0 hours). Extracted mRNA was assayed by qRT-PCR with TRPM2 specific primers. β-Actin housekeeping gene was used for normalization purposes. a) Relative TRPM2 expressions increased with asbestos exposure in both cell lines; however, a more consistent pattern was observed in LP9-Pass cells. b) Although asbestos exposure induced TRPM7 expression in each cell line, a better correlation with time and asbestos amount was obtained in LP9-Clone A cells. n=3, *p<0.05.

Based on these results, we selected the clone LP9-Clone A for the following experiments. It has been shown that TNF-α released by macrophages in vivo or exogenous TNF-α added in vitro is essential to induce transformation of HM. Therefore, to mimic the process of HM transformation and to verify the possible interaction with the ion channels, LP9-Clone A cells were exposed to TNF-α alone (10ng/mL), Crocidolite alone (10µg/cm²) or TNF-α and Crocidolite together for 12, 24, 48, 72 hours, and 1 week. RNA was extracted from cells after each treatment, and qRT-PCR was performed. It was observed that when cells were exposed to crocidolite and TNF-α together, the expression of TRPM2 ion channel
progressively increased until 72 hours of exposure, while after one week this effect was no longer detected (Figure 23a).

Figure 23 : Relative TRPM2 and TRPM7 expressions in LP9 immortalized primary cells exposed to asbestos with or without TNF-α. Immortalized human mesothelial cells were treated with TNF-α (10ng/mL) alone, Crocidolite (5 ug/cm²) alone or with TNF-α 24 hours prior to asbestos exposure for 12, 24, 48, 72 hours and 1 week. A time dependent increase in TRPM2 expression was observed for the asbestos and TNF-α co-treated cells for 12, 24, 48 and 72 hours. TRPM7 expression, on the other hand, showed the highest increase in 48 hours TNF-α and crocidolite co-treated cells, while other conditions presented a more inconsistent affect on its levels. n=3, *p<0.05.

7.2.2.3. Activity of TRPM2 and TRPM7 in mesothelial cells exposed to asbestos

It has been shown that TRPM2 and TRPM7 ion channels are involved in regulation of cell survival for their role in apoptotic cell death and cell proliferation, respectively. For example, the function of TRPM2 channel has been investigated in different cancer cells like glioblastoma and prostate cancer. Whereas the role of TRPM7 in proliferation of various cancer cells such as head and neck squamous carcinoma, gastric adenocarcinoma and breast cancer has also been demonstrated. In our previous experiments, we observed a change in the mRNA levels of TRPM2 and TRPM7 ion channels upon crocidolite and TNF-α exposure in immortalized mesothelial cells. This result was the first experimental clue for the correlation between the two ion channels and asbestos induced mesothelial cell transformation since TNF-α and crocidolite are shown to be able to transform primary mesothelial cells. In order to determine whether not only expression, but also activity of the ion channels can be also affected by the presence of carcinogenic finers, we performed Whole Cell Patch Clamp on the asbestos
exposed cells. This technique can measure the activities of the ion channels on the plasma membrane, evaluating Ca$^{2+}$ or Mg$^{2+}$ influx.

For the purpose of plasma membrane activity measurement, immortalized LP9-Clone A human mesothelial cells were cultured on coverslips until 70% confluent in 10% FBS containing DMEM. Cells were exposed to TNF-α (10ng/mL) or Crocidolite (5µg/cm$^2$) alone, or they were co-exposed to TNF-α and crocidolite together for 12 hours, 72 hours or 1 week. At the end of each treatment, TRPM2 and TRPM7 plasma membrane activities were measured for 10 individual cells in each condition and the average currents were plotted. Experiments were performed in the whole-cell configuration at 21-25 °C. All data were acquired with Pulse software controlling an EPC-9 amplifier (HEKA, Lambrecht, Germany) and analyzed using FitMaster (HEKA) and Igor Pro (Wavemetrics). Voltage ramps of 50 ms spanning the voltage range from –100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 200-500 s. Voltages were corrected for liquid junction potentials. Currents were filtered at 2.9 kHz and digitized at 100 µs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp. The ramp current amplitudes at –80 mV (inward current) were extracted from individual ramp data and displayed as current development over time.

Figure 24 shows that no significant difference was observed in TRPM2 plasma membrane activity between treated and untreated cells. Although an obvious change in the current was observed on the cells treated with asbestos and TNF-α for 72 hours or only with TNF-α for 1 week, their I/V values did not match with TRPM2 ion channel I/V value, that is why they were considered as artifact. A further investigation was required to identify what these current were. In contrast, at 12 hours and 72 hours exposures, TRPM7 plasma membrane activity increased the most when the cells were co-exposed to asbestos and TNF-α (Figure 25). However, in cells exposed to asbestos for 1 week, TRPM7 activity increased similarly with or without the presence of TNF-α. Exposure to TNF-α alone did not affect TRPM7 activity at any time point (Figure 25).
Figure 24. TRPM2 currents in mesothelial cells treated with asbestos. Immortalized LP9-Clone A human mesothelial cells were cultured on coverslips until 70% confluent in 10% FBS containing DMEM. Cells were then exposed to 5µg/cm² Crocidolite or 10ng/mL TNF-α alone, or they were co-exposed to 10ng/mL TNF-α plus 5µg/cm² crocidolite together for an additional 12 hours, 72 hours or 1 week. TRPM2 currents were measured using the Whole Cell Patch Clamp Method. Cells were kept in standard external solution supplemented with 1 mM Ca²⁺ and were perfused with standard Cs-glutamate-based internal solution supplemented with 1 mM ADPR in Ca²⁺ unbuffered conditions. Data were acquired using a 50 ms voltage ramp from −100 mV to +100 mV given at 0.5 Hz. Current amplitudes were extracted at −80 mV, normalized to cell size (in pF), averaged and plotted versus time (n = 10). Error bars indicate SEM. a) TRPM2 currents after 12h treatment b) 72 treatment and c) one week treatment.
Figure 25: TRPM7 Ion Channel Activity in mesothelial cells treated with asbestos. Cells were cultured on coverslips until 70% confluent in 10% FBS containing DMEM and further exposed to 5µg/cm² Crocidolite or 10ng/mL TNF-α alone, or they were co-exposed to 10ng/mL TNF-α plus 5µg/cm² crocidolite together for an additional 12 hours, 72 hours or 1 week. TRPM7 currents were measured using the Whole

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Cell Patch Clamp Method. Cells were kept in standard external solution supplemented with 1 mM Ca\(^{2+}\) and were perfused with standard Cs-glutamate-based internal solution supplemented with Cs-EDTA and Cs-BAPTA in unbuffered conditions. Data were acquired using a 50 ms voltage ramp from −100 mV to +100 mV given at 0.5 Hz. Current amplitudes were extracted at −80 mV, normalized to cell size (in pF), averaged and plotted versus time (n = 10). Error bars indicate SEM. a) TRPM7 currents after 12h treatment b) 72 treatment and c) one week treatment.

Although there was no significant change in the activity of TRPM2 ion channel in any of the treatments at any time point, the increase in the activity of TRPM7, especially when the cells were co-treated with TNF-α and crocidolite, suggested a role for TRPM7 in asbestos induced cell transformation. Moreover, the increased mRNA levels of TRPM2 upon the double exposure without any change in the activity might be due to a different intracellular localization of this ion channel (e.g. translocation to the cytoplasm) since Whole Cell Patch Clamp can only measure the activity on the plasma membrane.

7.3. Aim 3

To elucidate the role of TRPM2 and TRPM7 ion channels in MM

7.3.1. Rationale

The role of TRPM2 in various cancers such as glioblastoma and prostate cancer has been demonstrated. Activation of TRPM7 channels has also been shown to be critical for the growth and proliferation of human head and neck carcinoma cells and for the survival of human gastric adenocarcinoma cells. Up to date, no studies have focused on the role of TRPM2 or TRPM7 in MM cell proliferation, migration or wound healing properties. This prompted us to investigate the role of TRPM2 and TRPM7 ion channels in these activities of MM cells, by silencing the expression of the ion channel genes using shRNA.

7.3.2. Silencing of TRPM2 and TRPM7 gene by shRNA

The MM cell line PPM-Mill was selected for the preliminary experiments of gene silencing by shRNA expression, for its moderate levels of TRPM2 and TRPM7 proteins. The cells were infected with lentiviral particles expressing the gene encoding small RNAs able to impair TRPM2 or TRPM7 mRNA translation and the puromycin resistance gene, as described in Materials and Methods section. Lentiviral particles expressing “scramble” shRNA, made of scrambled sequences with the same composition of the specific shRNA, but not leading to specific degradation of any known cellular mRNA, were used as controls. Cells successfully transfected with shRNAs were selected upon resistance to 5 μg/mL puromycin selection.
7.3.2.1. TRPM2 Protein Expression in Silenced Mill Cells

The silencing of TRPM2 gene was monitored by Western blotting. Proteins from whole cell extracts were extracted with RIPA buffer, separated on SDS PAGE, transferred on membrane and probed with the specific antibodies. The results revealed 60% reduction of the TRPM2 protein (170 kDa) expression in PPM-Mill cells transduced with lentiviral TRPM2 shRNA (Figure 26), compared to uninfected cells. The cells transduced with lentiviral scramble shRNA showed only a 20% decrease in TRPM2 expression. Due to technical problems with the used antibody, the TRPM7 expression levels could not be detected.

Once stable cultures were established for the silenced PPM-Mill cells, experiments were performed to investigate the possible changes of three typical properties of tumor cells: viability, wound healing and migration.

Figure 26 : TRPM2 protein expression in PPM-Mill cells transduced with shRNA. Proteins were extracted with RIPA buffer from PPM-Mill cells that were transduced with lentiviral shRNAs. Western blotting was performed with the extracted proteins and the intensities of the bands around 170 kDa were analyzed by Image J software and normalized to the bands of loading control (Gapdh). PPM-Mill cells expressing shRNA targeting TRPM2 protein showed a 60% decrease in their TRPM2 protein expression, compared to untreated cells or transduced with scrambled shRNA.

7.3.2.2. Viability
In order to examine the role of TRPM2 and TRPM7 ion channels in MM cell viability, MTS assay was performed on scramble shRNA, TRPM2 shRNA, and TRPM7 shRNA transduced PPM-Mill cells, along with non treated cells. Experiments were done quadruplicates and the results were expressed as viability relative to untreated cells. Student test was applied to evaluate the significance of the differences between the groups. (Figure 27). A 1.5 fold increase in cell viability was observed when TRPM2 ion channel was silenced. A 1.2 fold increase was observed in cell viability of cells where TRPM7 was silenced, compared to untreated cells. However, the difference was not significant when compared to scramble shRNA expressing cells (1.1 fold). This result suggested a role for TRPM2 down regulation in the survival of mesothelioma, by possibly reducing the apoptotic cell death, while no significant effects was observed, by silencing TRPM7 ion channel.

![Graph](image)

**Figure 27 : Relative viability of PPM-Mill cells with and without shRNA treatment.** The same number of cells (3X10^3) for each silencing condition was cultured in 8 wells of a 96 well plate in 10% FBS containing DMEM with the selective antibiotic puromycin for 3 days. Relative cell viability values were evaluated by MTS assay. A significant 1.5 fold increase was observed in the viability of cells with silenced TRPM2 compared to wild type Mill cells. n=4, *p<0.05.
Since H₂O₂ is known to gate TRPM2 ion channel on the plasma membrane and its role in cell apoptosis also has been shown, we postulate a higher effect on cell viability of TRPM2 silenced Mill cells when H₂O₂ was present in the media. To test this hypothesis, 300 µM H₂O₂ was added to media where the cells were cultured. At the end of 2 days, the viability was measured with MTS assay. Experiment was done in triplicates and student t test was performed to evaluate the differences between treated and untreated cells (none). The differences were considered as statistically significant when p values were smaller than 0.05. In this condition, a 1.8 fold increase in viability was observed for cells expressing shRNA specific for TRPM2 (Figure 28). In absence of H₂O₂, the increase in the relative viability of TRPM2 silenced cells was 1.5 fold. This 20% increase in the viability rate further validated the role of TRPM2 ion channel in reactive oxygen species (ROS) induced cell death of Mill cells.

![Figure 28: Relative viability of PPM-Mill cells treated with shRNA in the presence of 300 µM H₂O₂.](image)

When shRNA treated cells were cultured in the presence of H₂O₂, the relative viability of TRPM2 silenced cells increased 1.8 fold compared to the ones with no treatment or transduced with lentiviral scramble shRNA. This rate was 20% higher than the one observed in the absence of H₂O₂, and it further validated the role of TRPM2 in ROS induced cell death in PPM-Mill cells.

7.3.2.3. Wound Healing
Another marker of cancer aggressiveness in vitro is the ability of the tumor cells to heal a gap made on the monolayer by mechanical injury ("scratch assay"). In order to test whether TRPM2 and TRPM7 ion channels have a role in healing ability of MM cells, we performed a "wound healing assay", or "scratch assay". We cultured the same number (5.0X10^5) of scramble shRNA, TRPM2 shRNA and TRPM7 shRNA cells, along with untreated cells in 6 well plates. On the cell culture was confluent, a scratch was made with a pipette tip and images were taken at increasing time points. When a complete healing of the gap in TRPM2 silenced cells was observed at 48 hours, the experiment was stopped and the images of the monolayers were taken (Figure 29). The differences between 0 hour gaps and 48 hours gaps were analyzed by Image J software. The results of the analysis were evaluated by student t test. The difference between treated and untreated cells was considered as statistically significant when p values was smaller than 0.05. A 3.5 fold increase in the healing ability of TRPM2 silenced cells was observed, as compared to untreated cells (Figure 30). This result highlighted a more aggressive behavior for tumor cells with TRPM2 ion channel silenced. A 1.8 fold increase in the healing ability of TRPM7 silenced cells compared to the untreated cells was also observed. However, although this increase was statistically significant, a similar effect was observed in lentiviral scramble shRNA transduced cells, reducing the relevance of the potential impact of TRPM7 silencing on this biological property.
Figure 29: Representative pictures of Wound Healing Assay for PPM-Mill Cells upon shRNA treatments. Cells were cultured in 6 well plates until they are confluent. A scratch was made and pictures were taken at 0 hour. At the end of 48 hours, a complete heal was observed for TRPM2 silenced cells and the pictures of the plates were taken. TRPM7 silenced cells showed no difference in healing compared to untreated cells.
Figure 30: Analysis of the wound healing assay in PPM-Mill Cells with different shRNA treatments. Photographs from Figure 27 were analyzed by Image J software. The differences in width between 0 hour gaps and 48 hours gaps were calculated as actual healing values. Relative healing rates were obtained by dividing the actual healing values of the treated cells by the actual healing value of non-treated cells. TRPM2 silenced cells had a significantly higher rate of wound healing compared to both non-treated and scrambled shRNA treated cells. TRPM7 silenced cells, on the other hand, had a higher rate of healing compared to the non-treated cells; however, since this effect was also observed in scramble shRNA treated cells, it may be an artifact generated by the silencing process. N=4, *p<0.05.

The wound healing assay involves the death of the mechanically injured cells located on the edges of the scratch area. The content of the cells damaged by the mechanical injury triggers the apoptotic cell death of the nearby bystander cells. Since TRPM2 ion channel is known to have a role in apoptotic cell death, we hypothesized that the observed higher healing rate of TRPM2 silenced cells might be due to the reduced rate of the apoptotic cell death of bystander cells that may become more resistant to apoptosis upon TRPM2 silencing. Subsequently, higher number of surviving cells may lead a quicker increase in the cell number of the population. Therefore, the observed faster healing process in TRPM2 silenced cells might be merely the result of an increase of the number of proliferating cells after the scratching. Because
it is known that TRPM2 ion channel contributes to the cell death through its Ca\textsuperscript{2+} regulating activity, we made the hypothesis that cells cultured in low Ca\textsuperscript{2+} conditions may drop their apoptosis rate to the basal levels and the effect of TRPM2 silencing can be suppressed.

In order to test this hypothesis, we cultured the cells in 6 well plates in the presence of a chelating agent. EGTA (Ethylene Glycol Tetra-acetic Acid) was chosen for this purpose since it specifically chelates Ca\textsuperscript{2+} ions. By adding 1.9 mM EGTA to 10%FBS containing DMEM media, we were able to bring down Ca\textsuperscript{2+} concentration from 1.95 mM to 55.7 \(\mu\)M. Once again, we scratched the surface of the confluent cultures and documented the wounded areas at 0 hour. At the end of 96 hours (Figure 31), cells were still not able to cover the healed area and this result suggested a role for Ca\textsuperscript{2+} in the healing process. More importantly, analysis of the pictures revealed no significant difference in healing between TRPM2 silenced cells compared to untreated cells when student t tes was applied, \(p\geq 0.05\) (Figure 32). The results supported our hypothesis that improved healing may be the consequence of the increased cell survival of the bystander cells.

![Images of cell cultures at 0 hours and 96 hours for both untreated (None) and TRPM2 silenced (shScrambled) conditions.](image-url)
Figure 30: Representative picture of Wound Healing Assay in TRPM2 silenced Mill cells in the presence of EGTA. Cells were cultured on a 6 well plate. 1.9 mM EGTA was obtained in the media to lower the Ca\(^{2+}\) concentration from 1.95 mM to 55.7 uM. A scratch was made and the pictures were taken as 0 hours. At the end of 96 hours, pictures were taken although complete healing of the wound has not been observed in any condition.

Mill Cells

![Image of Mill Cells with different treatments](image)

<table>
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<tr>
<td>sh-TRPM2</td>
<td>0.7</td>
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</table>

Figure 31: Analysis of wound healing assay in PPM-Mill cell with sh RNA treatments in low Ca\(^{2+}\) containing media. Pictures obtained from wound healing experiments were analyzed by Image J Software. No significant differences were observed between any of the treatments. n=4, p≥0.05.

7.3.2.4. Migration

Cell migration towards a chemoattractant has been widely used to monitor the tumor related behavior of a cell line. In this experiment, we cultured the same number of cells transduced with the different shRNA, in Transwell system chambers. Briefly, two compartments are separated by a porous membrane and cells, plated on the upper compartment, are challenged for migration toward a chemoattractant or upon a migratory stimulus (e.g. serum), through the porous membrane. Cells were grown in serum free conditions, but 10% FBS containing DMEM media was added to the upper chamber of the Transwell.
Since FBS contains growth factors, motility factors and cytokines acting as chemoattractants, cells move
towards its gradient. At the end of 24 hours, experiment was stopped and the Transwell membranes were
stained with HEMA3 Stain Set (Fischer Scientific Company LLC, Kalamazoo, MI). Cells grown in the
upper side of the Transwell membrane were erased with cotton swabs and photographs were taken to
identify cells attached to the lower side of the membrane (Figure 32). The pictures were analyzed by
Image J Software. Student t test was applied to the analysis results to evaluate the significance of the
difference in migration between treated and untreated (none) cells. The difference was considered to be
statistically significant when p values were smaller than 0.05. The analysis revealed that the differences in
migration rate between PPM-Mill cells treated with shRNA against TRPM2 and untreated PPM-Mill cells
were significant (Figure 33). Although there was a slight increase in the migration rate of PPM-Mill cells
transduced with shRNA against TRPM7, compared to control PPM-Mill cells, this increase was not
statistically significant.

![None sh-Scrambled sh-TRPM2 sh-TRPM7]

**Figure 32: Representative pictures of migration assay in PPM-Mill cells with shRNA treatments.**
The same numbers of the cells for each treatment were cultured in Transwell membranes. Cells were
stimulated to migrate toward the 10%FBS gradient. At the end of 24 hours, migrated cells were stained
and counted under the microscope. An evident faster migration was observed in TRPM2 silenced cells
compared to untreated cells, while scramble and TRPM7 shRNA transduced cells seemed to migrate
around the same speed as untreated Mill cells.
Figure 33: Analysis of migration assay pictures in PPM-Mill cells with sh RNA treatments. Pictures taken for the migration of the cells were analyzed with Image J software. TRPM2 silenced cells had nearly 3 fold higher rate of migration than the cells with no shRNA treatment. Scrambled and TRPM7 shRNA transduced cells; however, were not significantly different compared to the untreated cells. n=3, p<0.05.

While all three experiments suggested a more aggressive behavior of the TRPM2 silenced PPM-Mill cells compared to control cells, TRPM7 ion channel did not seem to influence these cell features significantly. Furthermore, the identification of TRPM7 by the antibodies available in the laboratory was unsuccessful. For this reason, in all further experiments performed with Ren cells (with moderate expression of the two ion channels), we excluded TRPM7 silenced cells and studied TRPM2 silenced cells only, compared to the scramble shRNA transduced cells.

7.3.3. Silencing of TRPM2 expression in Ren Cells

As described for PPM-Mill cells, Ren cells were also infected with lentiviral particles expressing the gene encoding small RNAs able to impair TRPM2 mRNA translation and the puromycin resistance gene. Lentiviral particles expressing "scramble" shRNA, made of scrambled sequences with the same composition of the specific shRNA, but not leading to specific degradation of any known cellular mRNA, were used as controls. Cells successfully transfected with shRNAs were selected upon resistance to 5 μg/mL puromycin selection.

7.3.3.1. IF Staining of TRPM2 Silenced Ren Cells
Silencing of TRPM2 gene in Ren cells was monitored by Immunofluorescence (IF) (Figure 34). The pictures of the stained cells were taken by using Leica Confocal Microscope. The presence of clustered shining dots was noticed in the nuclei of untreated Ren cells (indicated with a red arrow on the picture). The same amount of these clusters was also observed in scramble shRNA treated cells (indicated with a red arrow). Although these clusters were also seen in the nuclei of Ren cells treated with shRNA against TRPM2 mRNA, the amount of the visible clusters was remarkably lower compared to untreated and scrambled shRNA treated cells (indicated with a red arrow).

Figure 34: Representative pictures of the IF of Ren cells with or without sh-RNA expression. Control Ren cells and the cells transduced with lentiviral particles were porbed with fluorescein-labeled antibody. Pictures were taken under microscope and a visible reduction of TRPM2 protein expression was observed in the cells that were transduced against TRPM2 compared to control cells.

7.3.3.2. TRPM2 Protein Expression in TRPM2 Silenced Ren Cells

Although IF of Ren cells indicated a down regulation of TRPM2 protein when the cells were transduced with shRNA against TRPM2 mRNA, a Western blotting was also performed to confirm the results (Figure 35). With this technique, it was possible to evaluate the level of reduced expression by densitometric analysis of the blot image. This analysis revealed that Ren cells silenced by shRNA against TRPM2 mRNA had 90% lower protein expression compared to control Ren cells. Scramble shRNA treated cells showed an unexpected 50% reduction in their TRPM2 protein level. Although this reduction in scramble shRNA transduced cells was unusually high, TRPM2 silencing was 40% higher than the scramble shRNA treated cells, and the results comparing these cells could still be interpreted as a specific effect of TRPM2 silencing.
Figure 35: TRPM2 protein expression in REN cells transduced with sh RNA. Proteins were extracted with RIPA buffer from Ren cells that were transduced with lentiviral shRNAs. Western blotting was performed with the extracted proteins and the intensities of the bands around 170 kDa were analyzed by Image J software and normalized to the bands of loading control (Gapdh). Ren cells expressing shRNA targeting TRPM2 protein showed a 90% decrease in their TRPM2 protein expression, compared to untreated cells or transduced with scramble shRNA and 80% reduction compared to scrambled shRNA treated cells.

7.3.3.3. Viability

In order to examine the role of TRPM2 ion channels in Ren cell viability, MTS assay was performed on scramble shRNA and TRPM2 shRNA transduced Ren cells, along with control cells. Results were expressed as viability relative to control, non infected cells (Figure 36). Student t test was applied to evaluate the significance of the differences between untreated (none), scrambled shRNA treated and shRNA against TRPM2 treated cells. The difference was considered as statistically significant when p value was smaller than 0.05. A 1.1 fold increase in cell viability was observed when TRPM2 ion channel was silenced. A 1.05 fold increase was observed in cell viability of cells where scramble shRNA was used compared to untreated cells. In a parallel experiment, cell viability was represented by growth rates. In this experiment, the same numbers of TRPM2 shRNA transduced, scramble shRNA treated and control Ren cells were cultured in a 96 well plate. In three consecutive days, their viability was measured by MTS assay. For each condition, absorbance values of Day 1 were referred as 100%. The values of Day2 and Day3 were compared to this value and relative growth rate in each condition was calculated by dividing the values by the values of Day 1 and multiplying the result by 100. The results revealed that TRPM2 silenced cells had the highest growth rate, followed by the scramble shRNA transduced Ren cells. Control Ren cells displayed the lowest growth rate. Although the difference between scramble shRNA transduced
and not transduced cells was statistically significant, the fact that there was a even higher difference between control Ren cells and TRPM2 shRNA transduced cells and a statistically significant increase in viability in TRPM2 shRNA transduced cells compared to the scramble shRNA treated cells suggested a role for TRPM2 down regulation in the survival of mesothelioma, by possibly reducing the apoptotic cell death.

**Figure 36 : Viability of Ren Cells with shRNA treatments.** Scramble shRNA treated and TRPM2 shRNA treated cells were cultured in a 96 well plate along with the controls in 10% FBS containing media and in the presence of puromycin. On the third day after culturing, cell viabilities were measured by MTS assay. Relative viabilities of shRNA treated cells were calculated by referring to untreated cells as 100%. The results revealed an increase of relative cell viability in TRPM2 silenced Ren, although the difference was not as striking when compared to the scramble shRNA transduced cells. n=4, **p=0.002, ***p=0.001.

### 7.3.3.4. Wound Healing

As it was performed in PPM-Mill cells, Ren cells with different shRNA expression were also used to investigate the wound healing feature of the cells upon TRPM2 down regulation. In order to test the effect of TRPM2 silencing in Ren cells, the same numbers of scramble shRNA, TRPM2 shRNA transduced cells, and control Ren cells were cultured in 6 well plates The next day, a scratch was made with a pipette tip and 10ng/mL of Mitomycin C was added to the media. The pictures were taken at this time point and they were recorded as the data at 0 hours. When a complete healing of the gap in TRPM2 silenced cells was observed at 48 hours, the experiment was stopped and the pictures of the plates were taken (**Figure 37**). The differences between 0 hour gaps and 48 hours gaps were analyzed by Image J software. Analysis results were statistically evaluated by student t test. A statistically significant increase (p<0.05) in the healing ability of TRPM2 silenced cells was observed, as compared to untreated cells (**Figure 38**). Scramble shRNA transduced cells showed a decreased level of wound healing compared to control cells (represented with the asterix on the graph; **Figure 38**), and the difference between these two cell types was also statistically significant.
Figure 37: Representative pictures of wound healing assay in Ren cells with shRNA treatments.

Scramble and TRPM2 shRNA transduced cells were cultured in a 6 well plate along with untreated ones. Once the cells reached to 100% confluency, a scratch was made and the pictures were taken at 0 hour. When a complete healing was observed in TRPM2 silenced cells at 48 hours, the experiment was terminated and new pictures were taken.
7.3.3.5. Migration

Cell migration towards a chemoattractant has been widely used to monitor the tumor related behavior of a cell line. In this experiment, we cultured the same number of cells transduced with the different shRNA, in Transwell system chambers. Cells were grown in serum free conditions, then 10% FBS containing DMEM media was added to the chambers bearing the Transwell membranes. Since FBS contained growth and motility factors and cytokines, acting as chemoattractants, cells moved towards its gradient. At the end of 24 hours, experiment was stopped and the Transwell membranes were stained with HEMA3 Stain Set (Fischer Scientific Company LLC, Kalamazoo, MI). Cells grown in the upper side of the Transwells were erased with cotton swabs and pictures of the cells attached to the lower side were taken (Figure 39). When these pictures were analyzed by Image J Software, and the analysis results were evaluated by student t test, a significant difference ($p<0.05$) between untreated and TRPM2 down regulated cells was observed (Figure 40). Scramble shRNA treated cells; however, showed no difference in the ability of migrating compared to the untreated cells, suggesting a role for TRPM2 ion channel in vertical mobility of MM cells towards a chemoattractant.

**Figure 38**: Analysis of wound healing assay in Ren cells with shRNA silencing. Pictures taken for the wound healing assay were analyzed with Image J Software. A significantly higher healing rate for the cells with silenced TRPM2 was observed compared to both untreated and scramble shRNA transduced cells. $n=4$, $p<0.05$. 

**Figure 39**

![Graph showing the analysis of wound healing assay in Ren cells with shRNA silencing. The graph displays relative healing rate against different treatments: None, shScrambled, and shTRPM2. The y-axis represents the relative healing rate ranging from 0 to 200, while the x-axis lists the treatments. The graph shows a significant difference in healing rate between untreated and TRPM2 down regulated cells, as indicated by an asterisk (*) above the 'shTRPM2' treatment.](image-url)
Figure 39: Representative picture of migration assay in Ren cells with shRNA silencing. Scrambled and TRPM2 shRNA treated Ren cells were cultured in Transwell membranes along with the untreated cells. Cells were induced to migrate toward the 10% FBS containing medium. At 24 hour, membranes were stained with HEMA3 Stain Set, after removing cells from the upper side of the membranes.

Figure 40: Analysis of migration assay in shRNA silenced Ren cells. The stain from the migrated cells was taken into the solution by using 10% Acetic Acid. Absorbance was read at 595 nm and relative migration rates were calculated by considering the migration of untreated cells as 100. Relative migration rate was significantly higher in the cells with silenced TRPM2 compared to untreated cells. n=6, p<0.05.

7.4. Aim 4
To observe the localization of TRPM2 ion channel protein in HM and MM cells.

### 7.4.1. Rationale

TRPM2 ion channel has been mainly shown to localize on the plasma membrane and to have a role in cell death, it was also shown in prostate cancer cells that it could be localized in the nuclei and could have an opposite affect on cell survival. To check whether a nuclear localization also occurs in malignant mesothelioma cells, we performed immunofluorescent staining (IF) on HM and MM cells.

### 7.4.2. IF Staining of HM and MM cells

In order to determine cellular localization of TRPM2 ion channel, IF (Immunoflorescence) was performed on two primary mesothelial cells (HM10 and HM16; Figure 41) and three malignant mesothelioma cell lines (PPM-Mill, Ren and Con; Figure 42). Pictures were taken by using Leica Confocal Microscope at 488nm for TRPM2 (green) and at 560nm for Topro (blue) antibody. Since Topro is a nuclear protein specific antibody, nuclei for the cells appeared as blue dots in the pictures while TRPM2 protein was evidenced by its green color. Pictures were taken at each wavelength separately, then, they were merged by using Leica Microscope software to determine localization of TRPM2 protein. In the figures only the merged pictures were shown. Overlap of blue and green colors was read as the nuclear localization of TRPM2 protein. When the pictures were studied, the results pointed that TRPM2 was predominantly localized in cytoplasm in HM cells while it is mostly clustered in the nuclei of the MM cells studied. This observation raised the possibility that localization of TRPM2 ion channel might be an indication of mesothelial cell transformation. Moreover, it is also possible for TRPM2 ion channel to function differently depending on its intracellular location. Although the literature mainly focus on the ion gating activity of TRPM2 protein that is involved in cell death under ROS related stress conditions, one study questions its potential as a survival factor when located in the nuclei of the prostate cancer cells.
Figure 41: Merged IF pictures of HM10 and HM16 stained for Topro and TRPM2 protein. Mesothelial cells obtained from two individuals were stained against Topro and TRPM2 proteins. Blue color determines the location of the nuclei in cells. TRPM2 protein is represented by the green color in the pictures. Nuclear localization of TRPM2 proteins was observed where green and blue colors overlapped. Although a nuclear localization was visible for TRPM2 protein, its abundance in cytoplasm was evident.

Figure 42: Merged IF pictures of PPM-Mill, Ren and Con stained for Topro and TRPM2 protein. Three malignant mesothelioma cell lines were stained with Topro and TRPM2. Immunofluorescence pictures were taken under a confocal microscope. Blue color determines the location of the nuclei in cells. TRPM2 protein is represented by the green color in the pictures. Nuclear localization of TRPM2 proteins was observed where green and blue colors overlapped. Although a nuclear localization was visible for TRPM2 protein, its abundance in cytoplasm was evident.

8. DISCUSSION

8.1. Tumor Clonality and HUMARA Assay

The results of HUMARA assay and HUMARA-MSP assay in 6 MM tumor samples give a consistent indication for the polyclonal origin of mesothelioma. Since the normal and tumor cells were separated through Laser Capture Microdissection, possibility of cross-contamination of the cells was reduced. Even though we were confident that the risk of cross contamination was very low, we confirmed the reliability of our technique by doing a titration assay in which gradually changing percentages of biallelic and monoallelic DNAs were mixed. With this technique, we found that, in our methodology, up to 25% cross contamination could be ignored since 25% contamination remains unnoticed in the results. The monoallelic DNA mixture showed a monoallelic pattern when 25% of the mixture was bi-allelic. Similarly,
bi-allelic mixture showed a bi-allelic pattern when the percentage of monoallelic DNA present in the mixture was 25.

**8.2. The Peculiar Case**

Interestingly, the methods applied in our study were able to identify a particular condition in one MM specimen. In the sample labeled as L-III-18, a monoallelic pattern was observed for tumor and normal cells. Since normal cells also revealed such a pattern and this sample was obtained from a female patient, it was initially considered as an example of the condition called Skewed X Lyonization, which is seen in 5-20% of apparently normal women, and points out a non-random X-chromosome inactivation. However, a further investigation of this sample clarified that this was not the case for our sample. When we examined the untreated DNA obtained from the surrounding normal cells of L-III-18 tumor, we realized that the pattern revealed in this sample was quite unusual. Since it is expected for a normal female cell to have two X chromosomes and all present X chromosomes carrying HUMARA gene can be amplified by our assay when the sample is not digested, it was puzzling to see a single band for the PCR product of undigested DNA extracted from the normal cells. We considered two possible explanations for this observation. The first interpretation was that this female shows an X0 genotype for her sex chromosomes. Absence of Y chromosome in this genotype would determine the gender of the cells as female, and if the expression of the genes encoded in the present X chromosome is sufficient for the survival of the organism, this person would have a normal life and her rare genotypic condition would stay unnoticed. The second interpretation of these results was that the alleles of HUMARA gene encoded in two X chromosomes of this individual could be identical in the region we amplify. In this scenario, although two alleles exist and one is possibly methylated, we would not be able to detect it since our technique distinguishes the alleles by the number of CAG repeats that create a polymorphic region for the gene and subsequently change the molecular size of the PCR products. In another words, if the number of CAG repeats are the same in two alleles of HUMARA gene in this patient, then not only this sample would become uninformative for our purpose but also it would explain why we see a single band for the undigested normal cell DNA population because the PCR products of these two alleles would be indistinguishable by electrophoresis. In order to find out which explanation was the real one, we used the data obtained through the HUMARA-MSP performed on this sample. HUMARA-MSP uses two sets of primers; one is specific for methylated X chromosome and the other for unmethylated X chromosome. Because there would be no methylated X chromosome in the case of X0 genotype, we would not see any PCR product for the unmethylated primer if the first explanation was right. Instead, we saw a band for the unmethylated DNA in our experiments and the size of this product was very close to the size of methylated DNA. We considered the result of this experiment as the evidence that this individual has identical HUMARA genes in two alleles, which essentially means that one of the maternal alleles was identical to the one that is present in the father's genome. The chance of having two identical alleles for a
female offspring in such a family would be as high as 50%. As another possibility, if the mother of this individual had identical alleles and they were also at the same size as the father's allele, the chance of having identical alleles as a female offspring of this family would be 100%. Although the second possibility has a very low chance to exist, it is still possible. Moreover, if intra-familial marriages are common in the extended family of this individual, the possibility of having two identical alleles of the gene would become even higher compared to the situation in which having identical alleles was a pure coincidence. By taking advantage of the fact that this patient, L-III-18, is a member of a familial mesothelioma cohort which has been studied by our lab and therefore we have samples from several members of immediate and extended family relatives of the patient, we performed HUMARA assay on the undigested DNA extracted from the female siblings of this person to see if any of them showed a similar result. Although no female cousins or the daughters of the patient showed a single band for their mock digested DNA in HUMARA assay, luckily, the only healthy female sibling of L-III-18 that we have the DNA for presented a single band for her undigested DNA as well (data not shown). That was, to us, a strong support for the second explanation in which we considered the maternal and paternal alleles of HUMARA gene are being identical.

8.3. Possible Applications of Tumor Clonality

Determining the clonal pattern of mesothelioma may have a direct relevance to the mechanisms of carcinogenesis and might have also practical implications for both diagnosis and prognosis of disease progression. A possible polyclonal origin for mesothelioma may raise the possibility of the presence of small populations of distinct tumor cells in the neoplasm. Such clonal sub-populations might response differently to chemotherapeutic approaches, implying that multiple areas of the tumor may need to be screened for the molecular targets, by using a sensitive mutation detection method, before selecting a particular therapy.

In addition to possible clinical applications that could be assessed with the knowledge of polyclonality of a tumor, our results also give a fresh look for the initiation of the tumor formation. In today's scientific community, a tumor is considered to be a clonal population of cells. Since it is believed that mutations to transform a cell are rare events and therefore, it could happen to only a single cell in a relatively short time of a human life span, monoclonality of tumors is not only accepted but also expected. However, our results, along with a few other recent researches reviewed by Dr. Parsons challenge this belief. To my understanding, there is no reason not to think that multiple cells with the exact same genetic information in the same micro-environment can be transformed by a certain carcinogen, especially when we consider that the same carcinogens can cause mutations (and eventually transformation) in diverse micro-environments of several people with different genetic information. Notably, in case of cancers trigged by an environmental factor, such as asbestos exposure in mesothelioma, it would be highly likely for a tumor
to be originated from a number of cells/clones that were able to survive and proliferate under the stress inducing affects of carcinogens.

8.4. Clonality and Technical Limitations

Lastly, it needs to be underscored that HUMARA and HUMARA-MSP assays can only reflect the methylation status of X chromosome. If, in a tumor population, multiple cells that carry the same methylated X chromosome become transformed, the tumor will be determined as monoclonal even though it is derived from multiple progenitors. Additionally, if a female has more than two X chromosomes in her cells (e.g., XXX), then, the cells will inactivate all extra X chromosomes by keeping only one active in each cell. In this case, the tumor cell population will appear as polyclonal, regardless of its real clonal background.

8.5. Mesothelioma and TRPM2/TRPM7 Ion Channels

Our preliminary results on time- and dose- dependent increase of TRPM2 and TRPM7 expression in HM upon asbestos exposure suggested a role for these ion channels in cell survival and possibly in cell transformation for mesothelium derived cells. The fact that both ion channels reached the maximum expression levels only when TNF-α and crocidolite were both present strengthened the potential role of the channels in cell transformation, because TNF-α is known to have an important role in asbestos-induced oncogenesis. Especially the TRPM2 mRNA changing pattern points out a role for TRPM2 in asbestos-induced cell transformation by possible protecting the organism to activate the death pathways. In the experiment where LP9-clone A was co-exposed to asbestos and TNF-α, TRPM2 mRNA levels are upregulated by time 72 hours. When the treatment time reaches to its maximum duration (i.e., one week), then, TRPM2 mRNA levels go back to its basal values. This sudden down regulation could be explained by the fact that, in the presence of asbestos, most cells tend to die. The stress conditions created by the ROS production triggered by asbestos exposure, could lead to increased production of TRPM2 ion channel so that apoptotic and necrotic pathways become activated. However, since in every cell population, cells have slight variations in their expression of the genes, the cells that have the ability to keep their TRPM2 mRNA at the basal levels under stress inducing conditions, will be able to survive and become advantageous over other cells. If the stressful condition persists, this advantage might lead the low-TRPM2 expressing-cells to dominate the cell population. The cells that are able to escape from dying under stressful conditions might eventually transform and form a tumor. Thus, a seemingly advantageous feature of a group of cells in the short term can actually be disadvantageous to the organism in the long term.

8.6. TRPM7 Ion Channel and Human Mesothelial Cells
The up-regulation of TRPM7 activity measured on plasma membrane by whole cell patch clamp in TNF-α/Crocidolite co-exposed cells suggested a role in transformation of HM cells for this ion channel. The most striking observation in TRPM7 activity measuring experiment was that when the cells are exposed to asbestos or asbestos and TNF-α together for one week, results were very similar to each other. In another words, TRPM7 activity reached its maximum level when asbestos was present long enough and this activation seemed not to be affected by the presence of TNF-α. Here, we can hypothesize that the cells which were able to survive for one week under the selection of asbestos exposure could be the ones that were able to produce efficient amounts of TNF-α. This self-efficiency of the cells not only might have given them an advantage in terms of survival, but it might have also reduced their dependence on external TNF-α which increases the possibility for these cells to be transformed. This hypothesis could be tested by measuring the amount of TNF-α in the culture media and inside the cells at the end of one week exposure to asbestos with or without addition of external TNF-α. If the measured amounts of TNF-α are found to be similar in two conditions, theory becomes proven.

8.7. TRPM7 Ion Channel and Malignant Mesothelioma

Although the observations with immortalized HM cells and asbestos exposure suggested a role for TRPM7 activity in the asbestos induced cell transformation, its effects on already transformed cells was not significant. Our shRNA silencing experiments used against TRPM7 mRNA showed no significant difference in terms of viability, migration or wound healing features of MM cells between untreated, scrambled shRNA treated and TRPM7 silenced cells. However, there is one important fact to be considered when we interpret the results generated through TRPM7 shRNA treated cells, that is our inability of checking TRPM7 protein levels in untreated and shRNA against TRPM7 treated cells. The antibody we used against TRPM7 protein in previous Western Blotting experiments, produced not interpretable results for the down regulation of TRPM7 protein. Although the TRPM7 shRNA transduction process applied against TRPM7 mRNA was similar to what has been done against TRPM2 mRNA, which was successfully silenced, and puromycin selection ensured that the cells that were able to survive under this selection were efficiently transfected since MM cells normally die in the presence of puromycin and they are protected against it only when shRNA transduction was successful because the lentiviral particles used against TRPM7 mRNA also contained the gene that encodes N-puromycin acetyl transferase enzyme, which can recruit an acetyl group to N-terminus of the puromycin molecule and subsequently eliminate its killing effect on MM cells. However, a successful tranfection does not always mean a successful silencing. Therefore, without actual measurement of TRPM7 protein in the cells, our assumption of having TRPM7 down regulated MM cells remains as a hypothetical one. For this reason, the results of TRPM7 silencing should be cautiously evaluated and no conclusions can be drawn at this moment.
8.8. TRPM2 Ion Channel and Human Mesothelial Cells

The results obtained through the experiments on TRPM2, on the other hand, were in another direction. Although no change in the activity of TRPM2 on the plasma membrane was observed in any condition, reduction of TRPM2 protein expression in MM cells significantly altered their proliferation, migration and wound healing properties. The reason for not observing any variation in the activity of TRPM2 ion channel upon asbestos and TNF-α exposure at any time point might be due to the fact that only plasma membrane activity of the ion channels can be measured by the technique we performed here. As suggested in the literature for prostate cancer cells, TRPM2 can be localized exclusively in the nuclei and could have alternative functions that differ from the proposed roles of plasma membrane activity of the ion channel.

8.9. TRPM2 Ion Channel in Malignant Mesothelioma

Experiments with TRPM2 down regulated mesothelioma cell lines revealed an unexpected role for TRPM2 in the tumor cell behavior. Increased ability of viability, wound healing and migration pointed the possible tumor suppressor role for TRPM2 ion channel. The fact that the results obtained through migration and wound healing assays can be affected by the higher viability of the cells raised the possibility that our results from different assays might be pointing the same traits of TRPM2 silenced cells. In order to overcome this possible drawback, we decided to use Mitomycin C in the media that we used in wound healing and migration assays. Mitomycin C, as an agent that blocks the DNA synthesis, prevents the cells from being proliferated. This way, the higher number of migrated cells in TRPM2 silenced conditions, for example, could be related to only the higher vertical mobility of these cells, which can be seen as a sign of metastatic ability of tumor cells. Considering the fact that the results we obtained with the TRPM2 down regulated cells was reproducible in two different MM cell lines was a good indication that it might be a general trend in mesothelioma. If, with further research, such a link between mesothelioma and TRPM2 ion channel can be confirmed, targeting of TRPM2 gene and protein could be considered for a therapeutical approach.

With the knowledge of that TRPM2 is an ion channel that is known to be involved in reactive oxygen species (ROS) induced apoptotic cell death and asbestos is a ROS producing reagent, our observations on this ion channel brought a new point of view to the cell survival under asbestos exposure. Possibly, the cells that have lower levels of TRPM2 have a higher susceptibility to survive under stress, therefore prone to transform. Once they transform; however, TRPM2 activity appears to keep them in a relatively benign state. These two seemingly opposite roles of TRPM2 in the cancer related point of a cell behavior suggest a regulatory function for the levels of this ion channel. At its basal levels, TRPM2 seems to keep the cells
in an effective range of action. It neither allows the cells to die easily under stressful conditions nor lets them proliferate excessively. These observations provide a rationale for suggesting a regulator role for TRPM2 ion channel in the cell life span.

9. MATERIALS AND METHODS

9.1. HUMARA assay

A 50uL PCR reaction mixture containing 25 uL of HotStarTaq Master Mix Kit from Qiagen (Valencia, CA), 2.5 uL of DMSO, 40 pmol of each primer, and 100 ng of the DNA digested by HpaII from New England BioLabs (Ipswich, MA) or mock digested DNA is prepared. Primers used are 5’ACC GAG GAG CTT TCC AGA AT3‘ bearing a fluorescent FAM tag on the 5’end, and 5’TGG GGA GAA CCA TCC TCA C3’. Thermocycling involves of denaturation at 95°C for 10 minutes; 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 10 minutes. PCR products are separated on 3% Agorose gel after mixing with 6X loading dye and visualized on a UV transilluminator after incubation with ethidium bromide.

9.2. HUMARA-MSP Assay

Aliquots of 500 ng of DNA are modified with sodium bisulfate by using EpiTect Bisulfite Kit from Qiagen (Valencia, CA). Aliquots of 100 ng of modified DNA are added to 50 uL of PCR solution containing 25uL of HotStarTaq Master Mix Kit from Qiagen (Valencia, CA) and 2.5 uL of DMSO, 40 pmole of primer sets specific for unmethylated (U) or methylated (M) DNA. Sense Primers (Methylated : 5’CGAGCGTAGTATTTTTCGGC3’; Unmethylated : 5’GGTTGTGAGTGTAGTATTTTTTGGT3’) corresponded to a region at which DNA methylation is correlated with XCI. The antisense primer (5’TAAAAAAACCATCCTCACC3’) is designed corresponding to a sequence in a region containing no CpG dinucleotides. After denaturation at 95°C for 5 min, PCR amplification is performed with the following conditions : 40 cycles of denaturation at 95°C for 45s, annealing at 55°C and extension at 72°C for 1 min. Additional extension at 72°C for 15 min was adjusted at the end. PCR products were confirmed in 3% Agorose gels as the conventional HUMARA assay.

9.3. Semi-Nested HUMARA Assay

A 1ul of 1 to 100 diluted HUMARA assay PCR product is used in the 50 ul of PCR reaction mixture containing 25 ul of HotStarTaq Master Mix Kit from Qiagen (Valencine, CA) and 2.5 ul of DMSO and 40 pmol of each Semi-Nested primer. Semi-Nested primers are designed as forward : 5’ GAG CTT TCC AGA ATC TGT TC 3’ bearing a flourescent FAM tag on the 5’ end, and reverse : 5’ GAA CCA TCC TCA CCC TGC T 3’. Thermocycling consists of denaturation at 95°C for 10 minutes; 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 10 minutes.
PCR products are separated on 3% Agorose gel after mixing with 6X loading dye and visualized on a UV transilluminator after staining with ethidium bromide.

9.4. Cell Cultures

All types of cells used in this study are cultured in DMEM supplemented with 10% FBS. Primary human mesothelial cell cultures (HM) are obtained from patients who accumulated pleural fluid due to nonmalignant diseases. The mesothelial nature of isolated cells is assessed by immunostaining. They are used at passages 3-4. Immortalized human mesothelial cells (LP9) are from Dr. Rheinwald, Brigham and Woman Hospital, Boston, MA. MM cell lines derived from mesothelioma tumor tissues are also used in this study.

9.5. Asbestos Fiber Treatment

Crocidolite asbestos fibers are baked at 150°C for 18 hours, then suspended in Hank’s Balance solution, triturated 10 times through 18-22-gauge needles and autoclaved. For cell culture experiments, 5 or 10µg/cm² Crocidolite fibers are applied for 6 hours, 12 hours, 24 hours, 48 hours, 72 hours or 1 week.

9.6. Tumor Necrosis Factor-α (TNF-α) Treatment

TNF-α is from Sigma (St. Louis, MO). Cells are treated with TNF-α 10 ng/mL. Crocidolite exposed cells, are pre-treated 24 hours before asbestos exposure with 10 ng/mL of TNF-α. In long-term experiments, TNF-α 10ng/mL is added every two days.

9.7. Western Blotting

25 µg of cell lysates are separated on 4-12% acrylamide SDS-gel, transferred to PDF membranes and probed with TRPM2 or TRPM7 primary antibodies from Bethyl Laboratories (Montgomery, TX) and ProSci (Poway, CA) respectively. GAPDH or β-Actin primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) are used as loading control. Horseradish peroxidase conjugated secondary antibodies are from Pierce (Rockford, IL). Signal detection was performed by Enhanced ChemiLuminescence system (Thermo Scientific, Rockford, IL).

9.8. RNA Extraction

LP9 cells are cultured on T25 Flasks. Crocidolite is added at 90-100% confluency. At the end of the required exposure time, RNAs were extracted by using miRNeasy mini kit from Qiagen (Valencia, CA).
9.9. qRT-PCR

7900HT Fast Real Time PCR System was used for this experiment. Primers for TRPM2, TRPM7 and ACTB are from Qiagen (Valencia, CA; Cat. #: QT01870407, QT00082425, and QT01680476, respectively).

9.10. Whole-Cell Patch Clamp

Cells are cultured on cover-slips placed in a 6-well plate and exposed to TNF-α (10ng/mL), Crocidolite (10 ug/cm²) or both for 12 hours, 72 hours or 1 week. For electrophysiological TRPM7 measurements, TRPM7 conductance is maximized by using a tight-seal whole-cell configuration and Mg²⁺- and MgATP-free internal solution. Currents are elicited by a ramp protocol from -100 mV to +100 mV over 50 ms acquired at 0.5 Hz and a holding potential of 0 mV. Inward current amplitudes are extracted at -80 mV, outward currents at +80 mV, normalized to cell size and plotted versus time of the experiment. For electrophysiological TRPM2 measurements, intracellular potassium-based modified Ringer (K-Ringer) is applied. Intracellular Ca²⁺ is initially left unbuffered by omission of Ca²⁺ chelators. Currents are recorded for 200s by applying a voltage ramp spanning -100 mV to +100 mV and 50 ms length. Voltage ramps are acquired every other second (0.5 Hz). Current amplitudes are measured at -80 mV in each cell, averaged, normalized to cell size and plotted over the time of the experiment. For TRPM2 measurements intracellular Ringer's solution (in mM): 140 NaCl, 2.8 KCl, 1 CaCl₂, 2 MgCl₂, 10 glucose, 10 HEPES (pH 7.2 adjusted with NaOH). Standard intracellular solution contained (in mM): 140 Cs-glutamate, 8 NaCl, 1 MgCl₂, 10 HEPES (pH 7.2, 300 mOsm), 1 ADPR (for TRPM2) or 140 Cs-glutamate, 8 NaCl, 10 HEPES, 10 Cs-BAPTA, 5 Cs-EDTA (for TRPM7). Experiments were performed under Ca²⁺ unbuffered conditions.

9.11. shRNA Lentiviral Particles Transduction

HM and MM cells are cultured in a 12-well plate 24 hours prior to viral infection. When the plates are 50% confluent, the cell media is replaced with 5ug/ml of Polybrene consisting media. Adequate amounts of lentiviral particles constructed with TRPM2 or TRPM7 shRNA from Santa Cruz Biotechnology (Santa Cruz, CA) are added to the cell culture and incubated overnight. Medium is replaced with 1mL of complete medium without Polybrene, and cells are incubated overnight. Cells are split (1:3 to 1:5) and incubated for 24-48 hours. Stable clones are selected via Puromycin dihydrochloride selection and expanded.

9.12. Viability Assay
MM cells (5X10^3) are seeded in a 96 well plate and incubated at 37°C in a 5% CO_2 atmosphere for up to 72 h. Cell viability is measured by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay from Promega (Madison, WI).

9.13. Migration Assay

A Transwell system (8μm pore size, Corning Inc., Corning, NY) is used to evaluate cell migration. 100μl cell suspension (1X10^4 cells/ml) in FBS free medium is put in the upper compartment and 600μl DMEM medium containing 10% FBS is placed in the lower compartment. The chambers are incubated for 24 and 48 h at 37°C. The filters are fixed and stained using HEMA 3 staining kit from Fisher Scientific (Miami, OK). After gently rinsing with water, cells on the upper surfaces of filters are removed by wiping with a cotton swab. Cells remained on the lower surface of the filters are photographed under a microscope. To quantify the migratory ability, the crystal violet dye retained on the filters is resolved in 10% acetic acid and absorbance is colorimetrically measured at 595 nm.


MM and LP9 cells transfected with TRPM2 and/or TRPM7 shRNA are cultured and incubated for 24 hours. A clear area is then wounded with a micropipette tip. Migration of cells into wounded areas is evaluated with an inverted microscope and photographed at 24 and 48 h later. The number of cells migrated into the blank area of the plate is quantified using Image J software analysis. Mitomycin C with the final concentration of 5 µg/mL was obtained in the culture media to prevent the cells from proliferating.

9.15. Statistical Analysis

GraphPad Prism 5.0 software was used for the analysis of the results. Raw values were placed in columns as groups and student t test was applied.
10. REFERENCES


