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The role of the kinases of the nuclear factor-κB in malignant pleural mesothelioma

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ABSTRACT

Malignant pleural mesothelioma is a rare aggressive cancer that develops in the thin layer of tissue surrounding the lungs, known as pleural. The NF-κB transcription factor family is expressed in many types of tissues and plays a key role in triggering specific immune and inflammatory responses to environmental changes. NF-κB activates cellular behavior in many ways: inhibits apoptosis, induces increased cell proliferation, increases inflammation and immune response, etc. The function of the active NF-κB subunits that control all of the above depends on the activity of IKKα and IKKβ kinases in both NF-κB activation pathways. The purpose of this study is to determine the role of IKKα and IKKβ kinases in malignant pleural mesothelioma. For this purpose, gene expression of IKKα and IKKβ was silenced in AB2 and AE17 mesothelioma cancer cell lines and the potential changes in the proliferation rate of these cells were assessed in vitro. Our results demonstrate that kinase silencing, particularly IKKα, reduces statistically significantly the proliferation rate, which strongly indicates that IKKα kinase actively induces the aggression of cancer cells.
Το κακόηθες υπεζωκοτικό μεσοθηλίωμα είναι ένας σπάνιος επιθετικός καρκίνος που αναπτύσσεται στο λεπτό στρώμα ιστού που περιβάλλει τους πνεύμονες, γνωστό ως υπεζωκότα. Η οικογένεια μεταγραφικών παραγόντων NF-κB εκφράζεται σε πολλούς τύπους ιστών και παίζει βασικό ρόλο στην ενεργοποίηση συγκεκριμένων ανοσολογικών και φλεγμονώδων αποκρίσεων σε περιβαλλοντικές αλλαγές. O NF-κB ενεργοποιεί την κυτταρική συμπεριφορά με πολλούς τρόπους: αναστέλει την απόπτωση, επάγει την αύξηση του κυτταρικού πολλαπλασιασμού, αυξάνει την φλεγμονή και την ανοσοαπόκριση κ.α. Η λειτουργία των ενεργών υπομονάδων του NF-κB που ελέγχουν όλα τα παραπάνω εξαρτάται από τη δράση των κινασών IKKa και IKKB και στα δύο μονοπάτια ενεργοποίησης του NF-κB. Σκοπός της παρούσας μελέτης είναι ο προσδιορισμός του ρόλου των κινασών IKKa και IKKB στο κακόηθες υπεζωκοτικό μεσοθηλίωμα. Για το σκοπό αυτό, πραγματοποιήθηκε αποσιώπηση της γονιδιακής έκφρασης των IKKa και IKKB σε καρκινικές κυτταρικές σειρές μεσοθηλιώματος AB2 και AE17 και εκτιμήθηκαν in vitro οι πιθανές μεταβολές στο ρυθμό πολλαπλασιασμού των κυττάρων αυτών. Τα αποτελέσματα μας αποδεικνύουν ότι η αποσιώπηση των κινασών και ιδιαίτερα της IKKa μειώνει στατιστικά σημαντικά το ρυθμό πολλαπλασιασμού γεγονός που αποτελεί μια ισχυρή ένδειξη ότι η κινάση IKKa επάγει ενεργά την επιθετικότητα των καρκινικών κυττάρων.
<table>
<thead>
<tr>
<th>ACRONYMS</th>
<th>FULL NAME</th>
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<tbody>
<tr>
<td>CSCs</td>
<td>Cancerous Stem Cells</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>LCC</td>
<td>Large Cell Carcinoma</td>
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<tr>
<td>MPF</td>
<td>Megakaryocyte Potentiation Factor</td>
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<tr>
<td>MPM</td>
<td>Malignant Pleural Mesothelioma</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear Factor Kappa Beta</td>
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<tr>
<td>NLSs</td>
<td>Nuclear Localization Sequences</td>
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<td>NSCC</td>
<td>Non-Small Cell Carcinoma</td>
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<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
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<tr>
<td>SCLC</td>
<td>Small Cell Lung Carcinoma</td>
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<tr>
<td>SMRP</td>
<td>Soluble mesothelin-Related Peptide</td>
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<td>Squamous Cell Carcinoma</td>
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<td>SV40</td>
<td>Simian Virus 40</td>
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<td>TNF</td>
<td>Tumor Necrosis factor</td>
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<td>TNFR</td>
<td>Tumor Necrosis Factor Receptor</td>
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<td>TLR4</td>
<td>Toll-Like Receptor 4</td>
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Chapter 1: Introduction
1.1: Cancer

Cancer represents a huge burden on society in the most and least developed countries [1]. The incidence of cancer increases due to growth and aging of the population and the increasing prevalence of established risk factors such as smoking, obesity, lack of exercise and changes in the reproduction of urbanization and economic development [2].

Cancer is the name given to a number of related diseases. For all types of cancer, some of the body’s cells begin a continuous divide and diffuse into the surrounding tissue. Cancer can begin anywhere in the body, which is composed of trillions of cells. Normally, cells grow and divide to form new cells that the human body needs. If the cells grow too old or damaged, they die, and new cells take their place. But when cancer grows, the normal process is interrupted. As the cells become more abnormal, old or damaged cells will survive if they are to die and form new cells when they are not needed. Such additional cells can be separated without stopping and can be named as tumors [3].

The characteristic features of cancer include six biological properties during the growth of multi-stage human tumors. These include the maintenance of proliferative signaling, the prevention of the suppression of growth, the resistance to cell death, induced angiogenesis and activation of invasion and metastasis [4]. The main features are genomic instability, causing the genetic variation and inflammation, which promotes multiple sealing functions. The conceptual progress over the last ten years has allowed two new stamps in this catalog. Generality: reprogramming of the energy exchange and immune destruction prevent. In addition to cancer cells, tumors show another dimension of complexity that includes a repertoire recruited supposedly containing normal cells that carry on the properties of the seals that create the "tumor microenvironment".

There are more than two hundred different types of cancer, organized by the position in the body, with few exceptions, such as "HIV-related" or "Unknown Primary" tumours [5]. This body center system is most often laminated, for example, "Astrocytoma" is the subtype of brain tumors or the age of the patient, for example "leukemia in children" [6]. If each of these vital types has four subtypes, there are 800 subtypes of cancer. Cancer remains a killer disease in the world, despite the enormous amount of research and rapid progress achieved. According to the latest statistics, cancer is about 23% of all deaths in the United States and is the second most common cause of death after heart disease [7]. By 2020, it is expected that the
world population will have increased to 7.5 billion. Of this number, about 15 million new cases of cancer will be diagnosed and 12 million patients will die from it [8].

1.2: Lung Cancer

Lung cancer refers to malignant respiratory or pulmonary epithelial tumors. Carcinomas commonly affect the upper lobes of the lungs more frequently than the lower and the right lung more frequently than the left [9]. They are often diagnosed at late stage, when local invasion and metastases are already present [10]. This has encouraged the development of screening programs in an attempt to diagnose at an earlier stage [11].

Primarily lung and bronchial cancers are synonymous with lung cancer, but in the incidence of increasingly peripheral adenocarcinoma.

There has been a significant progress in understanding the biology of cancer, due to the large development of understanding tumor biology and pathogenesis. Acquisition of somatic mutations essentially acts as a guard in lung carcinogenesis, growth and division of tumor cells [12]. Pilot mutations in specific histological types of molecular recognition of lung cancer can provide a positive response to targeted therapy. The essence of personalized medicine is the adaptation of individual lung cancer treatment according to the classification and accurate information of histopathological biomarkers. Therefore, the characterization of the histological type of lung cancer plays a more important role in the multidisciplinary approach to the diagnosis and treatment of lung cancer.

Lung cancers are traditionally divided into non-small cell carcinoma (NSCC) and small cell carcinoma (SCLC), with the former accounting for 80% of the cases and the latter accounting for the remaining 20%. SCLCs are treated in most cases, whereas NSCCs are managed by a combination of surgery and adjuvant therapy. Recognition of the diversity of NSCC has led to its subclassification, culminating in the 2004 [13] and 2015 World Health Organization (WHO) classifications [14]. Major types of NSCC include adenocarcinoma, squamous cell carcinoma (SSC), and large cell carcinoma (LCC) [15]. Significant update in lung cancer classification has occurred for lung adenocarcinomas based on better understanding of tumor biology. This update is manifested by streamlined classification for small biopsies and cytology specimens, with special emphasis on separating adenocarcinomas from the rest of the lung cancers in order to effectively screen cases responsive to current mutation-
1.3: Malignant pleural mesothelioma

Malignant mesothelioma has been shown to be an aggressive tumor that is at the same time quite resistant to existing therapies. Its rapid rise is almost the case in most countries. The main risk factor due to the appearance of this tumor is exposure to asbestos, but [16] a virus, simian virus 40 (SV40) could play a role. Patients usually have dyspnea and chest pain with pleural effusions.

It arises primarily from the surface serosal cells of the pleura and peritoneum [17]. It can also develop from the serosal surfaces of the pericardium or the tunica vaginalis [18]. Up to 80% of all cases are pleural in origin and are defined as malignant pleural mesothelioma (MPM).

The pleura consist of a thin and elastic membrane covering the entire inner surface of the chest cavity. It is almost continuous, so it is a swelling pouch with a small amount of lubricant for the smooth movement of the thoracic cage, lungs, heart and internal organs. The metaphysical pleura covers the chest wall, the mesothelioma, the heart and the diaphragm and the visceral pleura covers the lungs [19].

Malignant pleural mesothelioma (MPM) remains a disease that causes death in 2,000 to 3,000 individuals each year in the United States. However, MPM remains a serious problem as the global incidence of the disease continues to increase. According to recent estimates, more than 5,000 new cases per year are expected to occur in Western Europe, with more than a quarter of a million deaths expected over the next 40 years. In Japan, the maximum incidence rate is predicted in 2025, and 103,000 deaths are expected over the next 40 years [20].

MPM is mainly observed in males (male to female ratio, 5: 1) [21]. According to surveys conducted, the risk increases with age (the mean age of diagnosis is 72 years in the United States ranging from 45 to 85 years) [22].

It is a fact that occupational exposure to asbestos is the most important risk factor for the MPM [23]. The development of this disease is more related to age groups between 20 and 60 years [24].
Asbestos is classified into two main families, the serpentines and the amphiboles. The serpentines consist of one type, chrysotile, with characteristic short, curly fibres, also called “white asbestos” due to its colour, which accounts for 95% of asbestos in commercial use. The amphiboles, with straight, longer fibres, include crocidolite or “blue asbestos”, amosite, tremolite, actinolite and anthophyllite, and a thorough review of their physical and biological properties has recently been published [25].

Histologically, malignant mesotheliomas can show an epithelial morphology (malignant mesothelioma epithelial type), a fibrous morphology (malignant mesothelioma fibrous type, also called sarcomatoid type), or a combination (mixed type or biphasic malignant mesothelioma). Most malignant mesotheliomas (50% to 60%) are of the epithelial type, approximately 10% are sarcomatoid, and the rest are biphasic malignant mesotheliomas. In general, at least 10% of the tumor must have a fibrous (or epithelial) component for the malignant mesothelioma to be classified as biphasic. It is a fact that all malignant mesothelioma showing both morphologies can be characterized as biphasic although one of the two morphologies may represent a small number of cancer cells. Correct identification of the histological type is necessary as it provides enough information as tumors with sarcomatoid morphology are particularly resistant to therapies and the average survival time observed is less than 1 year from diagnosis. On the other hand, most epithelial tumors, particularly well differentiated variants, are associated with prolonged survival 2 years after diagnosis. According to studies, there are unusual morphological variants, but rare, and some malignant mesothelioma cannot be subcloned histologically and should be called poorly differentiated malignant mesothelioma [26].
Figure 1.1: Histology of mesothelioma. (A) Epithelioid mesothelioma: note tubular structures and malignant mesothelioma cells showing a hob-nail morphology with bland nuclei and abundant cytoplasm; (B) biphasic malignant mesothelioma, showing a nest of mesothelioma cells with epithelial differentiation within the sarcomatoid component; (C) sarcomatoid mesothelioma: positivity for pankeratin and rare foci of cells suggestive of epithelioid differentiation indicated the diagnosis; (D) high-grade sarcoma: highly aggressive tumor that is vimentin positive but negative over 15 different immune stains for other markers and without distinctive electron microscopic features. In this case, the diagnosis of mesothelioma could not be confirmed [27].

The optimal serum biomarker for mesothelioma would predict the development of mesothelioma in asbestos-exposed subjects, differentiate mesothelioma from benign pleural disease or metastatic cancer, be useful for all pathologic subtypes, and correlate with the extent of disease in order to monitor treatment response and predict prognosis. Several small, retrospective series have evaluated the following three new biomarkers: soluble mesothelin-related peptide (SMRP), megakaryocyte potentiation factor (MPF) and osteopontin [28].

The optimal serum biomarker for mesothelioma could predict the development of mesothelioma in people who were exposed to asbestos, and to differentiate benign pleural mesothelioma from metastatic disease or cancer.

1.4: NF-κB and cancer

The experiments of Dr. Ranjan Sen led to the discovery of the NF-κB transcription factor [29]. In mammalian cells, the NF-κB / Rel family contains five members: RelA (p65), c-Rel, RelB, NF-κB1(p50, p105) and NF-κB2(p52). These proteins have a structurally conserved 300 amino acid sequence called the REL region, which contains the domains of dimerization, nuclear localization and DNA binding. Three of the family members, RelA, c-Rel and RelB, have a C-terminus activation field. NF-κB1 / p105 and NF-κB2 / p100 are the inactive precursors of the p50 and p52 proteins, respectively [30].

These proteins are located in the cytoplasm in an unstimulated state. The proteolytic treatment removes the C-terminal inhibitor domains, and in this way the proteins are introduced into the nucleus [31]. P50 and p52 typically form homodimers or heterodimers with one of the three proteins having a terminator region. RelA and p50 exist in a wide variety of cell types, while c-Rel expression is limited to hematopoietic cells and lymphocytes. The expression of RelB is limited to very specialized positions, such as thymus, lymph nodes, and Peyer's patches. Although each NF-κB dimer has a different DNA binding affinity for Kb positions.
carrying the consensus sequence GGGRNNYYCC (R, purine: Y, pyrimidine: N, any base) [32], their functions often overlap. NF-κB complexes consisting exclusively of members of the family lacking transactivation domains, such as p50 homodimers, are believed to impose transcriptional repression [33].

NF-κB is expressed in the cytoplasm of virtually all cell types, where its activity is controlled by a family of regulatory proteins, called inhibitors of NF-κB (IK). IκBa, IκBb, IκBe, and Bcl-3, members of the IκB family, commonly have 6 to 7 ankyrin repeats, which are 33 amino acid sequences that mediate binding to NF-κB dimers. The unprocessed NF-κB1/ p105 and NF-κB2/ p100 proteins also contain ankyrin repeats at their C-termini, which cause them to be included in this inhibitory family. IκB proteins were originally thought to retain NF-κB dimers in the cytoplasm by masking their nuclear localization sequences (NLSs). Recently observations, however, have indicated that both IκBa and IκBe shuttle between the nucleus and cytoplasm within NF-κB-IκB complexes. These complexes are capable of displacing NF-κB from target DNA sites and transporting it back to the cytoplasm [34]. The expression of IκB proteins is regulated by NF-κB; this feedback regulation is believed to contribute to the rapid shut down of NF-κB signaling. IκBb expression, however, is not regulated by NF-κB. Instead, IκBb is constitutively retained in the cytoplasm, indicating that it is not involved in the autoregulatory loop terminating NF-κB signaling. As seen for NF-κB, each IκB family member has both distinct and redundant actions [35].

Overall, two main NF-κB-activating pathways exist in cells [36]. The canonical pathway is induced by most physiological NF-κB stimuli. Signals emanating from cytokine receptors, such as the tumor necrosis factor receptor (TNFR) and interleukin 1 (IL-1) receptor (IL-1R), antigen receptors and pattern-recognition receptors, including Toll-like receptor 4 (TLR4). The canonical pathway is defined as dependent on IKKβ and NEMO and leads mainly to phosphorylation of IκBα and nuclear translocation of mostly p65-containing heterodimers [37]. In contrast, the noncanonical pathway depends on IκKα-mediated phosphorylation of p100 associated with RelB and leads to partial processing of p100 and the generation of p52/RelB complexes. Noncanonical signaling is induced by specific members of the TNF cytokine family, such as CD40 ligand, BAFF and lymphotoxin-β2 [38].
Direct mutations in NF-κB signaling genes have so far been detected primarily in lymphoid malignancies [40]. Relief and point mutations of RelA were detected in human B-cell lymphomas [41] such as Hodgkin’s lymphoma and to a lesser extent also in T-cell lymphomas, reflecting the direct oncogenic potential of NF-κB proposed by the initial discovery of oncogenic RelA homologue V-Rel. Additionally, mutations of other NF-κB members, including Bcl-3 and c-Rel [42], have been detected in B cell leukemia and various types of B-cell lymphomas, respectively [43].

A recent research on breast cancer revealed mutations in the NFKB1 gene, IKK2 upstream kinase, as well as IkBa and IkBe inhibitors [44]. Studies of transgenic rats indicate that there is a direct contribution of NF-κB to the development of various solid tumors. The best example to be studied is inflammation associated with colorectal cancer, where NF-κB induced by IKK2 in intestinal epithelial cells has an essential role in tumor formation. In addition, NF-κB activity mediated by IKK2 in myeloid cells in the tumor environment contributes to its progression through the induction of secretion of cytokines and growth factors [45].

**Figure 1.2**: Canonical and alternative pathway of NF-κB activation [39]
A different type of cancer associated with inflammation is hepatocellular carcinoma, a form of liver cancer, which can occur after viral hepatitis or after liver damage caused by carcinogens. Interestingly, the role of NF-κB in hepatic cancer appears to be highly dependent on the exact mechanism of cancer growth. Tumors associated with chronic inflammation appear to require NF-κB in hepatocytes as an anti-suppression survival factor. However, in some chemically induced types of liver cancer, hepatocellular NF-κB, in contrast, acts as a tumor suppressor, as shown by rat studies with hepatocyte-specific deletion of IKK2 or NEMO and application of diethyl-nitrosamine (DEN) Carcinogenic substance [46]. However, this type of cancer still requires NF-κB inside Kupffer cells, that is, liver macrophages, which are essential for IL-6 secretion and activation of STAT3 in adjacent hepatocytes.

Another cancer dependent on NF-κB activity is melanoma as it could be shown that HRAS-mediated onset of oncogenesis requires IKK2-mediated activation of NF-κB in melanoma models in rats [47], and even for lung cancer it could be considered that IKK2 and NF-κB are vital cofactors [48]. Generally, aberrant NF-κB activity appears to have an important role as a cofactor in solid tumors acting as a survival factor for a transformed cell, which otherwise could become apoptotic or senescent. This increased essential activity of NF-κB is usually achieved by the continuous release of cytokines from the macrophages into the tumor microenvironment. However, there is still a mystery in the relationship between solid tumors and adjacent macrophages. It seems that time is vital for the interaction between cancer cells and macrophages. An initially inflammatory environment that is activated by the tumor could induce TNFα and IL-1β secretion from macrophages, while prolonged tumor growth and chronic inflammation may lead to a shift towards the M2-type TAM.

In addition to the role of NF-κB in the survival of cancer cells or the response of immune system cells to cancer, NF-κB has recently been shown to be activated in cancerous stem cells (CSCs), where it can promote a pro-inflammatory environment, inhibit apoptosis and stimulate cell proliferation. Cancer stem cells contain only a small subpopulation of cancer cells and are believed to mediate tumor growth and resistance to chemotherapy [49].
Chapter 2: Materials and methods
2.1: Immunohistochemical staining

Immunohistochemistry is a technique for the detection of antigens in tissue sections using the interaction of specific antibodies on antigens. This technique starts with the preparation and purification of the tissue in sequential xylene solutions, xylene / alcohol, reduced strength alcohol (100% -96% -80%) and Tris-HCl buffer [Tris-HCl buffer (TBS ) M, pH = 7.6]. One of the challenges of immunohistochemistry is the development of methods that reverse the changes that occur during stabilization. Stabilization modifies the quaternary structure of proteins (antigens), making them often undetectable by specific antibodies. Detection of antigens can be significantly improved by treatment with antigen epithelial resuscitation agents such as heat treatment in the presence of 10 mM sodium citrate buffer, pH = 6. Also, endogenous peroxidase activity has been found in many tissues and can be detected by the reaction with constant chromogenic substrate. To eliminate endogenous peroxidase activity pre-incubation of the tissue with 0.3% hydrogen peroxide is essential. Background immunoreactivity can be specific or non-specific. This type of background staining is usually uniform and can be reduced by blocking these sites with saline solution (TBS / BSA, 3%). The tissue is next incubated with the primary antibody at the appropriate dilution specific for the antigen of interest. For optimal signal intensity incubation in the presence of a second antigen that binds with the first antibody is needed. Finally, detection of peroxidase binding sites is performed by applying a solution of H₂O₂ and diaminobenzidine 3,3-tetrahydrochloride (DAB), which gives the characteristic colorimetric product. Here we used for this the DAKO EnVision™ and HRP Dakocytomation polymeric label, (USA).The tissues are stained with Harris hematoxylin (Surgipath) as a counterweight and dehydrated in a series of increasing concentration alcohols (80% -96% -100%). This is followed by the clarification of the tissue in soluble xylene and covering of the slides with coverslips (Entellan).

2.2: Recovery of Cryopreserved Cells

The cell solution in the frozen vial needs to be warmed as rapidly as possible and then immediately combined with complete culture medium and seeded into an appropriate culture dish. Viability for most cells declines and reaches a nadir at 24 hours post-thaw. Most, if not all, of this decline appears to be due to apoptosis (as opposed to necrosis) induced by the stress of the cryopreservation process. After this time point, cells begin to recover and enter exponential growth. The steps for cell defrost include:
1. Prepare a culture dish so that it contains at least 8 mL of the appropriate culture medium equilibrated for temperature and pH.

2. Remove the vial from the liquid nitrogen freezer and thaw by gentle agitation in a 37°C water bath (or a bath set at the normal growth temperature for that cell line). Thaw rapidly until ice crystals have melted (approximately 2 minutes).

3. Remove the vial from the water bath and decontaminate it by dipping in or spraying with 70% ethanol. Follow strict aseptic conditions in a laminar flow tissue culture hood for all further manipulations.

4. Unscrew the top of the vial and transfer the contents to a sterile centrifuge tube containing 1 mL complete growth medium. Remove the cryoprotectant agent by gentle centrifugation (5 minutes at 300 × g). Discard the supernatant, taking care not to disturb the soft pellet, and resuspend the cells in 2 mL of complete growth medium. Pipette gently to loosen the pellet and break apart clumps. (If the cells normally grow as clusters, avoid over-pipetting during resuspension.) Transfer the cell suspension into the medium in the culture dish and mix thoroughly.

5. Examine the cultures after 24 hours and subculture as needed.

2.3: Cryopreservation Procedure

To prepare cells for cryopreserving:

1. Prepare a freeze medium consisting of 45 ml FBS and 5 ml DMSO.

2. Collect cells by gentle centrifugation (5 minutes at 300 × g) and resuspend them in the freeze medium at a concentration of 1 × 10⁶ to 5 × 10⁶ viable cells/mL. Continue to maintain the cells in culture until the viability of the recovered cells is confirmed.

3. Label the appropriate number of vials with the name of the cell line and the date. Then add 1 mL of the cell suspension to each of the vials (depending upon the volume of the vial) and seal.

4. Allow cells to equilibrate in the freeze medium at room temperature for a minimum of 15 minutes but no longer than 40. This time is usually taken up in dispensing aliquots of the cell suspension into the vials. After 40 minutes, cell viability may decline due to the DMSO.

5. Place the vials into a pre-cooled (4°C), controlled-rate freeze chamber and place the chamber in a mechanical freezer at −70°C (or colder) for at least 24 hours.
6. Quickly transfer the vials to a liquid nitrogen or –80°C freezer

2.4: ShRNA lentiviral particles transduction

Day 1: Plate target cells (100,000 cells/well) in a 6-well plate 24 hours prior to viral infection. Add 2 ml of complete optimal medium (with serum and antibiotics) and incubate cells overnight. The cells should be approximately 50% confluent on the day of infection (Day 2).

Day 2: Prepare a mixture of complete medium with Polybrene® (sc-134220) at a final concentration of 5 µg/ml. Remove media from plate wells and replace with 1 ml of this Polybrene/medium mixture per well (for 6-well plate). Thaw lentiviral particles at room temperature and mix gently before use. Infect cells by adding the shRNA Lentiviral Particles to the culture. Swirl the plate gently to mix and incubate overnight. The amount of viral particles to use varies greatly depending on the characteristics of the cell line used.

Day 3: Remove the culture medium and replace with 2 ml of complete medium (without Polybrene). Incubate the cells overnight.

Day 4: Select stable clones expressing the shRNA via Puromycin dihydrochloride (sc-108071) selection. For puromycin selection, use an amount sufficient to kill the non-transduced cells. Puromycin concentrations ranging from 2 to 10 µg/ml are usually sufficient, but a puromycin titration is recommended when using a new cell line. Replace medium with fresh puromycin (10 µg/ml)-containing medium.

Day 5: After 72 hours, we observe the percentage of expression of GFP to ensure about the efficiency of transduction.

Day 6: Replace medium with fresh puromycin every day, until resistant colonies can be identified. Pick several colonies, expand them and assay them for stable shRNA expression.

We used copGFP Control Lentiviral Particles (sc-108084) from Santa-Cruz Biotechnology as a control to monitor and optimize transduction efficiency, thus assuring satisfactory levels of targeted shRNA-knockdown. After transduction, they allow confirmation of the transduction efficiency of the Lentiviral Particles in a target cell population by expression of GFP detectable by fluorescence microscopy after 72 hours.

Cells stably expressing copGFP may be isolated via puromycin selection. The initial concentration of puromycin was 10 µg/ml, then we reduced gradually to the level of maintenance of the cells (1 µg/ml).
2.5: Cell Culture Preparation and Cytoplasmic and Nuclear Protein Extraction

1. For adherent cells, harvest with trypsin-EDTA and then centrifuge at 500 × g for 5 minutes.
2. Wash cells by suspending the cell pellet with PBS.
3. Transfer 1-10 × 10^6 cells to a 1.5mL microcentrifuge tube and pellet by centrifugation at 500 × g for 2-3 minutes.
4. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible.
5. Continue in protein extraction using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions.

2.6: Western blot protocol

Western blotting is an important technique used in cell and molecular biology. By using western blot, we are able to identify specific proteins from a complex mixture of proteins extracted from cells. The technique uses three elements to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody.

1. Gel preparation: These consist of 2 kinds of gel, the stalking gel (1/3) in which proteins are packed and the running gel (2/3) in which the proteins run and separate.
2. Protein samples can be quantitated either with Bradford or with a quantitation with NanoDrop. Calculate 70mgr of protein in a total volume of 20μl with 5 μl Laemli buffer 5x.
3. Place the samples in a heat block at 95 ° C for 5 minutes.
4. Centrifuge samples.
5. Place gels on device electrophoresis.
6. Load the specimens and run on stalking gel at 40 V and increase the voltage to 100V into the running gel.
7. I cut the PVDF membrane which is electroplated and will stick to it negatively charged proteins without creating background.
8. Place the membrane in 100% methanol for 5 minutes.
9. Fill the methanol and put 1x buffer for transport over 20 minutes on a moving
platform.
10. Prepare the transfer of the proteins by adding to the transfer device.
11. Run at 4 °C for 1 hour at 120V.
12. After transferring, block in milk (5 grams of Regilait in 100 ml PBST (1lt 1xPBS + 1ml Tween20)) for 1 hour.
13. Add antigen according to concentration in solution identical to the one blocked (3ml). Incubate overnight at 4 °C.
14. Wash with PBST for 5 minutes
15. Add the secondary antibody to the appropriate concentration for 1 hour at room temperature.
16. Wash 4 times in PBST for 5 minutes each.
17. Dry with either air or Whatman paper.
18. Cover membrane with 1ml of chemiluminescent solution.
19. Dry membranes with Whatman.
20. Fold the membrane and place it on the display cassette.
21. Use developer and fixer solutions for film display.

Solutions and Reagents

**Loading buffer: 2x Laemmli buffer**

260mM Tris ph=6.8 (2.6 mL),

5%SDS (2,5ml , 20%),

10% 2-mercaptoethanol (1ml)

14.8% glycerol (2,8ml 50%)

0,001% bromophenol blue

**10x Transfer Buffer (1Lt)**

58 gr Tris base

29gr Glycine

1xTransfer Buffer we will add in the following order:

700ml H2O + 100ml 10x TB + 200ml MetOh.

**Running gel 8% (10ml)**

4.6ml H2O

2.7ml acrylamide mix
2.5ml 1.5M Tris-Cl ph=8.8
0.1ml 10%SDS
0.1ml APS
0.004 ml TEMED
Stalking gel (6ml)
4.1ml H2O
1ml acrylamide mix
0.75ml 1M Tris-Cl ph=6.8
0.06 ml 10%SDS
0.06ml APS
0.006 ml TEMED
10x Running Buffer
250mM Tris=30.3gr Tris Base
1.92mM Glycine=144gr
V_{\text{final}}=1lt
1xRunning= 100ml 10x R.B.+890ml H2O+ 10ml 10%SDS

2.7: RNA extraction

1) Lyse cells directly on the culture dish. Use 1 ml of the TRI Reagent per 10 cm² of glass culture plate surface area. After addition of the reagent, the cell lysate should be passed several times through a pipette to form a homogenous lysate.

2) Phase Separation: To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Add 0.2 ml of chloroform per ml of TRI Reagent used. Cover the sample tightly, shake vigorously for 15 seconds, and allow to stand for 5 minutes at room temperature. Centrifuge the resulting mixture at 12,000 × g for 15 minutes at 2–8 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).
3) Transfer the aqueous phase to a fresh tube and add 0.5 ml of 2-propanol per ml of TRI Reagent used in Sample Preparation, step 1 and mix. Allow the sample to stand for 10 minutes at room temperature. Centrifuge at 12,000 × g for 10 minutes at 2–8 °C. The RNA precipitate will form a pellet on the side and bottom of the tube.

4) Remove the supernatant and wash the RNA pellet by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI Reagent used in Sample Preparation, step 1. Vortex the sample and then centrifuge at 7,500 × g for 5 minutes at 2–8 °C.

5) Briefly dry the RNA pellet for 10 minutes by air-drying or under a vacuum. Do not let the RNA pellet dry completely, as this will greatly decrease its solubility. Do not dry the RNA pellet by centrifugation under vacuum (Speed-Vac®). Add an appropriate volume of formamide, water, or a 0.5% SDS solution to the RNA pellet. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55–60 °C for 10–15 minutes. Final preparation of RNA is free of DNA and proteins. It should have a $A_{260}/A_{280}$ ratio of ≥1.7.

2.8: RT-PCR and Q-PCR

The RNA isolated from the cell cultures was processed by the reverse transcription polymerase chain reaction (RT-PCR) method. This method, exploiting the ability of the reverse transcriptase enzyme to transcribe RNA into DNA, uses the mRNA as template and transcribes it into the corresponding cDNA. In this way we can estimate the amount of mRNA of the gene of interest. The transcription reaction of RNA was performed using Supercritical III (ThermoFisher Scientifc, Cat. No. 18080085) and oligonucleotides.

Quantitative PCR (qPCR) is a fast and reliable method which allows quantification of specific target sequences. Real-Time quantitative PCR allows to measure the amount of product throughout the reaction by monitoring the fluorescence increase of a fluorescent substance.

All reactions were performed on a StepOnePlus cycler (Applied Biosystems, Carlsbad, CA) using SYBR Green, KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Cat. No. KK4604), as the fluorescent substance. This substance is excited by 497 nm wavelength radiation and emitting at 520 nm. It is noted that this substance does not fluoresce when it is free in solution. However, its incorporation into DNA in its synthesis results in the production of fluorescence. Therefore the intensity of the fluorescence is proportional to the product produced.
2.9: MTT assay

The yellow tetrazole MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells where enzymes consume it leaving equal amounts of NADH and NADPH.

In this process, purple crystals are formed which can be dissolved with acidified isopropanol and quantitated by photometry. The larger the number of cells, the higher the absorption values. For each cell type, the linear relationship between the number of cells and the generated signal allows an accurate quantitative estimate of the proliferation rate.

For this purpose the cells were cultured in 96-well culture plates with complete nutrient at a concentration of 3,000 cells per well (100 μl of nutrient medium). Every 24 hours for four days on the dishes was placed 10μl of MTT solution (5mg / ml) and after four hours the precipitate was dissolved in acidified isopropanol (0.1N HCl) and finally absorbance was calculated at 492nm.
Chapter 3: The role of IKKα-IKKβ in MPM
3.1: Determination of the expression of NF-κB kinases IKKα and IKKβ in MPM mice models of disease.

To assess whether IKKα and IKKβ are expressed on MPM and pulmonary tissue, we performed immunohistochemistry in tissue sections from mice models with malignant pleural mesothelioma.

![Images of immunohistochemistry](image)

**Figure 3.1:** Photos from immunohistochemistry in normal lung and malignant pleural mesothelioma from BALB/C mice with intrapleural infusion of AB2 cells and from C57BL/6 mice with infusion of AE17 cells for expression of kinases IKKα and IKKβ.

Both kinases IKKα and IKKβ exhibit immunoreactivity in MPM that arises from the parietal or is infiltrating the pulmonary tissue in both cases of mice models of the disease, BALB/C mice intrapleuraly injected with AB2 mesothelioma cells, or C57BL/6 mice intrapleuraly injected with AE17 mesothelioma cells.
3.2: Determination of activation of the classical and alternative pathway of NF-κB in mouse and human mesothelial cancer cells

The overexpression of the two kinases in tissues derived from mice models of MPM intrigued us to characterize the activation of NF-κB in different human and mouse cell lines of MPM. This was achieved by performing western blotting in protein extracts from these cell lines. The following figure illustrates the activation of the classical and alternative pathway of NF-κB in cytoplasmic and nuclear protein extracts from human (MSTO-211H and ZL34) and mouse (AE17 and AB2) malignant mesothelial cells, indicating a more alternative manner of activation for the nuclear –κB factor.

![Western blotting showing cytoplasmic and nuclear expression of all the subunits of NF-κB in human and mouse cell lines derived from malignant pleural mesothelioma.](image)

In cytoplasmic protein extracts we observe that:

- RelA (65 KDa) is expressed in ZL34 cells
- P50 (105 KDa) is expressed in AE17 cells
- P50 (50 KDa) is expressed in AE17 cells
- RelB (68 KDa) is expressed in ZL34, AE17 and AB2 cells
- P52/P100 (54 KDa) is expressed in Met5A, AE17 and AB2 cells
In nuclear protein extracts we observe that:

- RelA (65 KDa) is expressed in ZL34 cells
- P50 (105 KDa) is expressed in MSTO-211H and ZL34 cells
- RelB (68 KDa) is expressed in ZL34, AE17 and AB2 cells
- P52/P100 (54 KDa) is expressed in Met5A, AE17 and AB2 cells

These results demonstrate that NF-κB is mainly activated in these cell lines by the alternative pathway which is regulated by IKKα and less by the canonical which is regulated by both IKKα and IKKβ.

3.3: Studying the role of NF-κB kinases in mesothelioma cancer cell lines:

3.3.1: Gene expression silencing of IKKα and IKKβ with shRNA Lentiviral transfection.

In order to determine the transfection efficiency of the lentiviral transduction performance, we detected the expression of GFP in the fluorescence microscope for two mice mesothelioma cell lines, AB2 and AE17.

![Image](image.png)

**Figure 3.3**: Representative images of expression of GFP in mesothelial cell lines (AB2, AE17) using a fluorescence microscope
The expression of GFP in a field of a well plate with cultured mesothelioma cells is represented on the left figures while on the right is shown the same field on the visible light. We observe that the transfection rate reaches 90% in both cell lines indicating that our transfection efficiency is highly elevated.

To further validate the IKKa and IKKβ gene silencing levels, we used specialized primers of IKKa and IKKβ mRNAs, and we evaluated the gene silencing efficiency by the Real-Time quantitative PCR.

![Bar plots represent IKKa and IKKβ gene silencing levels in mesothelial cell lines (AB2, AE17)](image)

**Figure 3.4:** Bar plots represent IKKa and IKKβ gene silencing levels in mesothelial cell lines (AB2, AE17)
As we can see in the above diagrams, we can determine the levels of IKKα and IKKβ mRNAs produced in the parental mesothelioma cancer cells (AE17, AB2) and their “daughter cells” after gene silencing.

As we can see for AE17 cells, there is a statistically significant difference between AE17shcontrol and AE17shIKKα (p<0.0001). Similarly, there is a statistically significant difference between AE17shcontrol and AE17shIKKβ (p=0.0037) which means that in both cases gene silencing was successful.

As we can see for AB2 cells, there is a statistically significant difference between AB2shcontrol and AB2shIKKα (p=0.0001). Similarly, there is a statistically significant difference between AB2shcontrol and AB2shIKKβ (p=0.0217) which means that in both cases gene silencing was successful.

3.3.2: The role of IKKα and IKKβ kinases in cancer cells proliferation ability.

Finally, after validating the successfulness of gene silencing in mesothelioma cancer cells, the cells were cultured in 96-well culture plates in an initial number of cells (2000 per case) as described in chapter 2 in order to estimate the effect of the kinases gene silencing in the cells proliferation dynamic.

Figure 3.5: Diagrammatic illustration of cell proliferation rates (AE17shcontrol, AE17shIKKα, AE17shIKKβ and AB2shcontrol, AB2shIKKα, AB2shIKKβ) in relation to time.
In both cases of cell lines (AE17 and AB2) we observed that in the third and fourth day their daughter cells, not expressing IKKα and IKKβ exhibited a decrease in cell proliferation. In particular, a small but statistically significant decrease was found in AE17 mesothelioma cells not expressing IKKβ in relation to AE17 cells expressing IKKβ while a higher statistically significant decrease in cell proliferation was observed in AE17 mesothelioma cells not expressing IKKα. Furthermore, AB2 parental and daughter cells exhibited a same pattern where IKKβ gene silencing had a smallest but statistically significant effect in cell survival and proliferation while IKKα gene silencing effect was statistically significantly higher.

Summarizing, the rate and in general the dynamic of cancer cells to proliferate decreases in time upon gene silencing of IKKα and IKKβ, indicating their important role for the malignant phenotype of these cells.
Chapter 4: Discussion
As it has already been highlighted, malignant pleural mesothelioma is a rare aggressive cancer mostly and not only presented at the late stages of lung cancer disease. The NF-κB transcription factor family is expressed in many types of tissues and is implicated in several types of cancer including MPM, as well as in most of the hallmarks that characterize a malignant phenotype. The function of the active NF-κB subunits that control all of the above depends on the activity of IKKα and IKKβ kinases in different ways, in both NF-κB activation pathways.

The purpose of this study is to determine the role of IKKα and IKKβ kinases in malignant pleural mesothelioma.

The main findings of this study are summarized below:

- IKKα and IKKβ kinases are both expressed in MPM mice models (BALB/C mice that have been intrapleurally injected with AB2 mesothelioma cells and C57BL/6 mice that have intrapleurally been injected with AE17 mesothelioma cells).

- NF-κB canonical and alternative pathway activation is differentially present in several human and mice malignant mesothelioma cell lines.

- Both IKKα and IKKβ kinases play a crucial role for the survival and the proliferation rate of malignant mesothelioma cell lines in vitro.

Not many studies are found in literature about the role of IKKs in MPM. Most of them are focusing mainly in human MPM cell lines and less in murine as well as in the role of NF-κB in this phenomenon by studying the expression of the subunits that participate in the canonical activation pathway.

In human mesothelial cells exposed to asbestos, activation of NF-κB through TNF-α triggering has been well studied. However, these studies are limited to the canonical pathway and not to the alternative. In particular, an increased nuclear translocation of RelA has been observed in increased TNF-α cell exposure, using the Bay II-7082 inhibitor which is an inhibitor of IκB phosphorylation. Therefore, the scientists believe that TNF-α protects cancer cells from dying by activating the NF-κB classical pathway [50]. Although this approach is limited only to RelA expression in these treated cells it partly comes in contrast with ours. Our study is performing a more complete characterization of the activation of NF-κB in different but of the same disease cell lines. It is noteworthy that TNF-α activated NF-κB has also been observed in rat cell lines too.

In another study, where human mesothelioma cell lines were used and inhibition of IκB function in the NF-κB classical pathway was performed a group of
Scientists found a decrease in RelA of NF-κB, as well as a decrease in the rate of proliferation of cancer cells [51]. This study lacks in the investigation of the alternative activation pathway, upon this treatment. In concordance with our own results in human cell lines, we initially did not notice RelA being expressed in our cell lines except ZL34. Additionally, IKKβ (which is implicated in the canonical activation pathway) silencing caused a reduction in the rate of proliferation of our murine cancerous cell lines. Since p50 is expressed in both MSTO-211H and ZL34 and only to RelA in ZL34 we believe that probably, if we perform gene silencing for IKKβ in our human cell lines, the same phenotype might be observed.

Furthermore, it is worth noting that comparing the present with other studies we suspect that either inhibiting gene expression or using a kinase inhibitor significantly affects the cell proliferation rate, which means that kinase IKKβ plays an important role in the survival of mesothelioma cancer cells.

A similar study has shown that another drug, Disulfiram-Cu (DSF) suppresses the survival, proliferation and invasion signaling of AB12 and human MPM cell lines H2373, H2452, H2595, H2714 and H2461 by deactivating NF-κB. In addition, this study shows that using the drug increases IκB expression levels, meaning that RelA and p50 cannot enter the nucleus, thus deactivating the NF-κB pathway [52]. In combination with our own results, it is likely that this drug might also affect IKKα and IKKβ kinases and perhaps it should be further studied about its effects in the role and mechanisms of act of the two kinases involved in the canonical activation pathway.

Moreover, in another study 3 different types of mesothelioma cell lines have been used, Met5A without crocidolite asbestos treatment, Met5A with crocidolite asbestos treatment and NCI-H28, a cell line derived from epithelioid malignant pleural mesothelioma. In this study the investigators tried to use all cell types in the generation and progression of MPM, from benign to malignant and performed protein analysis. They found several molecular NF-κB-related complexes activated further proving its significant role in MPM formation. Although Nf-κB was found to be important in MPM progression, RelA had decreased expression in the cell nucleus [53]. However, it was found that the subunits of the alternative pathway, ie p52 and RelB, are expressed indicating that the alternative pathway is activated in these cancer cells and helps to create and increase mesothelioma progression.

As mentioned above, there is no extensive reference to the NF-κB alternative pathway activation in MPM in the literature. We believe that a more extent study of its differential activation is necessary to find biomarkers for disease diagnosis, as well as a more detailed study of the action of specialized inhibitors that suppress NF-κB activation in order to achieve a possibly more successful treatment.
In conclusion, NF-κB is highly important in MPM and as shown in the present study both IKKα and IKKβ play a crucial role in it. Although our results indicate their significance more in depth studies both in vitro and in vivo are required for the exact mechanisms by which IKKα and IKKβ are implicated or promote this phenomenon.
REFERENCES


