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Epithelial to Mesenchymal Transition (EMT) in human mesothelial cells exposed to asbestos fibres.

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Abstract

Introduction. Asbestos is the common name for a group of hydrated silicates, with a fibrous appearance, which are found commonly in nature. Fibres exposure increases the risk of inflammatory disease development, such as pleural effusion, pleural plaques and asbestosis (asbestos induced pulmonary fibrosis). Moreover, clinical features of malignant pathologies associated with asbestos exposure are Malignant Mesothelioma (MM), a fatal tumor arising from mesothelial cells, and lung cancer. Both asbestosis and MM clinical features have been associated to the Epithelial to Mesenchymal Transition (EMT), a series of biochemical and morpho-structural events implicated in fibrosis development and in cancer progression. In epithelial cells, the EMT drives cells to lose their epithelial characteristics in favor of a phenotype of mesenchymal nature. Some EMT markers confirm the EMT event, such as the downregulation of E-Cadherin and the overexpression of Fibronectin. Conditions of hypoxia and numerous growth factors are implicated in EMT induction. Among these factors, TGFβ plays an important role in pathological processes of the lung, such as fibrosis and cancer, and it is involved in cell proliferation, differentiation, apoptosis, cell cycle, and tumor development. TGFβ promotes the EMT event and asbestos has been demonstrated to induce TGFβ production, so as MM have been associated to EMT and its markers modulation.

Aims. In the present study we evaluate if chrysotile asbestos exposure could induce EMT in human mesothelial cell line via TGFβ secretion, trying to demonstrate a possible correlation to fibrosis or MM development.

Results. Chrysotile asbestos induces EMT in human mesothelial cells (MeT-5A) by downregulation of epithelial markers E-Cadherin and β-Catenin, and an increase of mesenchymal markers (α-SMA, Fibronectin, Vimentin, Metalloprotease), via TGFβ downstream effector SNAIL.

We demonstrated that EMT event evoked by chrysotile exposure is mediated by increased TGFβ secretion, so driving the EMT in mesothelial cells. Moreover, we observed also an increase of TWIST and ZEB-1 factors, also associated to EMT, via E-Cadherin inhibition, and both TGFβ mediated.

Conclusions. Taken as a whole, chrysotile induces EMT in mesothelial cells via TGFβ pathway/SNAIL. SNAIL, so as TWIST and ZEB-1, has been shown to be overexpressed in MM, thus it is conceivable to hypothesize that asbestos could mainly induce MM development or metastasis via EMT.

Content

1. Introduction

1. Introduction

Asbestos

"Asbestos" is the generic name referred to a group of naturally occurring hydrated silicate fibres, whose tensile strength and resilient structural and chemical properties are ideally suited for various construction and insulating purposes. Asbestos includes the serpentine mineral chrysotile, also known as "white asbestos" (Figure 1.1), and the amphibole minerals (amosite, crocidolite). Amphiboles are distinguished from one another by their chemical composition, and in natural samples the composition varies with respect to major and trace elements (such as iron). Asbestos fibres tend to possess good strength properties, flexibility, excellent thermal properties, adsorption capacity, and resistance to chemical, thermal and biological degradation. These amazing properties made asbestos fibres very desirable for a lot of industrial applications, with production and consumption of asbestos that have declined in recent years due to the introduction of strict regulations governing exposure and/or outright bans on exposure. In particular, chrysotile asbestos still accounts for 90% or more of commercially used asbestos fibres worldwide. Although its use is banned in the European Union and in many other countries, chrysotile is still widely mined and exported to developing countries (*Burki, 2010*). Asbestos exposure is associated with pulmonary fibrosis (asbestosis) and tumor diseases such as lung cancer and malignant mesothelioma (MM). Asbestos use and its ability to cause respiratory and malignant diseases of the lungs, the pleura, the peritoneum and the alimentary tract can be traced throughout all history. However, despite the epidemiological evidences, a huge increase in its use was registered at the beginning of the XX century. With the increasing use of asbestos materials, an increased number of asbestos-related diseases emerged. Epidemiological studies concerning asbestos exposure have been started since the very early beginning of the XX century, when a suspicion of association between asbestos exposure and lung cancer was reported by Lynch andSmith(1930).Thefirstcompletedescriptionofasbestosisappearedin1927among

asbestos workers (*; McDonald, 1982*) and studies of occupational exposure to asbestos occupied the whole XX century and are still current because of the long latency period of asbestos-related diseases.

Actually, no single mechanism fully accounts for all the complex toxic and carcinogenic effects caused by asbestos (*Kamp and Weitzman, 1999*). Aware of the previously mentioned difficulties in the determination of asbestos toxicity, a lot of works in literature focused on the ability of many different features of the fibres to affect the biological response following asbestos exposure: the shape, the dimension, the presence of iron, the redox state and the hydrophobicity of the surface, the ability to generate reactive oxygen species, the bio-persistence (*WHO, 1986*). The evidence of asbestos biological effect is mainly related, in literature, to the presence of long fibers, and only more recently the scientific attention shifted to nanosized fibers, that can be recovered in exposed worker and in people exposed to environmental pollution and that can naturally occur in the environment (*Miserocchi, 2008*).

Figure 1.1: Chrysotile asbestos (Serpentine)

Factors involved in asbestos toxicity

Inhalation is the primary route of asbestos exposure. The degree of penetration in the lungs is determined by the fibre diameter, and thin fibres having the greatest potential for deep lung deposition (*NTP, 2005*). Families of asbestos-workers may be exposed via contact with fibres carried home on hair or on clothing. In studies of asbestos concentrations in outdoor air, chrysotile is the predominant fibre detected.

Exposure by inhalation, and to a lesser extent ingestion, occurs in the mining and milling of asbestos (or other minerals contaminated with asbestos), the manufacturing or use of products containing asbestos, construction, automotive industry, the asbestos-abatement industry (including the transport and disposal of asbestos-containing wastes).

Toxicity of asbestos fibres depends on different asbestos properties, such as chemical composition, fibres-derived free radicals and release of inflammatory molecules and other mediators (surface area and mineral contaminants), the size of fibres (pulmonary deposition and clearance). In particular, surface properties are able to affect the fibre translocation, cellular activation, phagocytosis and production of free radicals (Reactive Oxygen Species, ROS, and Reactive Nitrogen Species, RNS). Moreover, the presence of iron can be considered a crucial variable in asbestos related toxicity and related effects could result in deregulation of normal iron metabolism by proteins within the cell, resulting in iron-catalyzed oxidation of biomolecules (*Gazzano, 2007*). Iron mobilization (Figure 1.2) is also associated to many different pro-oxidant cellular effects, including lipid peroxidation (*Ghio, 1998*), DNA oxidation (*Aust, 1999*), release of pro-inflammatory cytokines (*Qi, 2013*), cell death, DNA strand breaks and DNA oxidation (*Hardy,1995*).

Also the biopersistence is a very important condition for asbestos toxicity. In the lung, fibres tend to persist in the site of deposition and clearance mechanisms are mainly related to the shape

and size of particles. In particular, in the case of asbestos fibres the chemical composition is not sufficient to describe their toxicity. Moreover, natural asbestos fibres can be contaminated by different ions from metals, including Al, Fe, Ti, Cu, Na, K e Ca. Metal impurities are able to affect the hydrophobicity of fibre surface, and the ability to produce free radicals by hiding the crystalline surface and masking fibre toxicity (*Fubini and Hubbard, 2003*).

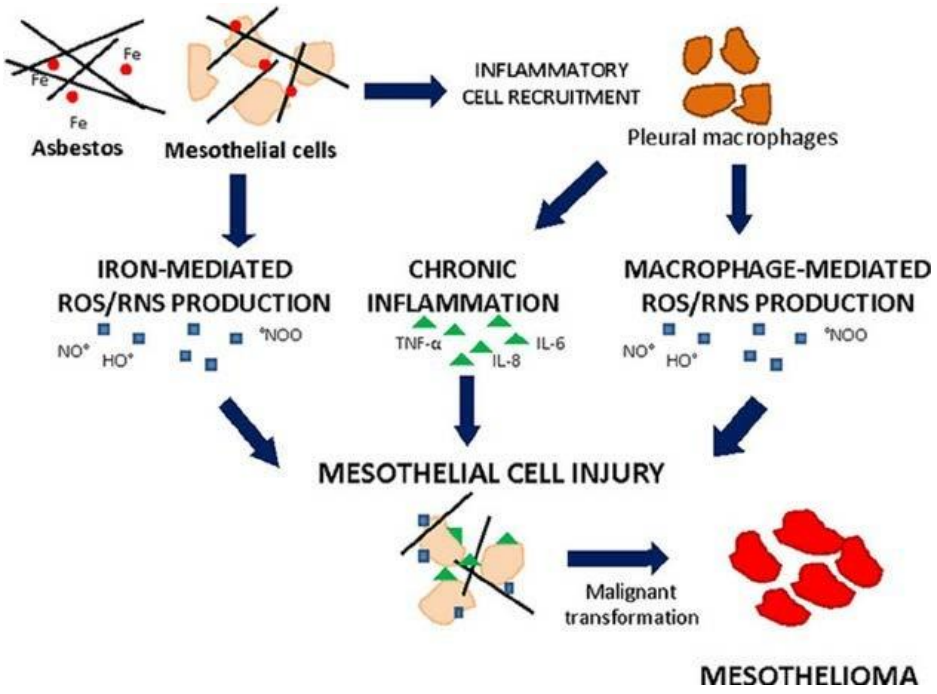


Figure 1.2: Asbestos-induced cell injury leading to mesothelioma. Mesothelial cells exposed to iron- and macrophage-mediated ROS/RNS production and inflammatory cytokines can cope cell injury and undergo malignant transformation giving rise to mesothelioma

Asbestos related diseases

Studies have linked more than a dozen different diseases to asbestos exposure. Many of these diseases, such as malignant mesothelioma and lung cancer, have a confirmed relationship with asbestos exposure. Others, such as Chronic Obstructive Pulmonary Disease (COPD) or kidney cancer, are not directly caused by asbestos, but researchers suspect that exposure can increase a person's risk for developing them (*Candura SM, et al.,2016*).

Asbestos-related diseases can range from mild and benign to malignant and life-threatening. Malignant mesothelioma (MM) tend to be more rare than benign illnesses, yet some of the benign conditions are just as serious as cancer. Asbestosis is a severe condition, leading to more deaths per year between 1999 and 2001 than asbestos-induced gastrointestinal cancer did during the same time frame.

Not everyone who is exposed to asbestos will get one of these diseases. However, exposure does increase the risk to develop one or more of these conditions. This elevated risk lasts for decades after exposure.

Asbestos-related benign diseases

Asbestos-related benign diseases are slightly more prevalent than related malignancies. In one study on 231 asbestos-exposed workers, 99 of them developed at least one benign lung abnormality (*Dumortier P. et al., 2003*).This elevated incidence rate may partially be related to the amount of exposure necessary for the conditions to develop. Actually, benign diseases often require less exposure. While cancers have a characteristic latency period of several decades, non-cancerous illnesses can arise within only a few years (*Koskinen K. et al., 2003*).

Asbestosis: Asbestosis is a benign yet deadly lung disease that is characterized by severe fibrosis and inflammation of lung tissue. For seven of the eight years between 2000 and 2007, asbestosis was an underlying or contributing cause of death for more than 1,400 people in the United States (*Biscaldi G. et al.,1999*).

Pleural Effusions: Pleural effusions are fluid build ups between the pleural layers. They may develop independently, but they are often a symptom of late-stage mesothelioma.

Approximately 1.5 million cases of pleural effusion are diagnosed each year in the United States (*Rascu A, et al., 2016*).

Pleural Plaques: Pleural plaques arise fairly frequently after asbestos exposure. These calcified build ups on the pleura are not considered a severe health issue. In asbestos-exposed populations, the incidence of pleural plaques ranges from 5 to nearly 50 percent, depending on factors like duration and concentration of asbestos exposure (*Cottin V. et al., 2008*).

Pleuritis: Asbestos fibres can cause excessive inflammation of the pleura, known as pleuritis. In geographic areas with higher incidence of mesothelioma, approximately 10% of people diagnosed with pleuritis of unknown cause go on to develop mesothelioma (*RascuA. et al., 2016*).

Asbestos-related cancer diseases

Malignant Mesothelioma (MM). Asbestos exposure is responsible for nearly all cases of MM, which represents so the signature for this asbestos-related cancer. It is also one of the most deadly diseases, causing between 2,000 and 3,000 deaths each year only in the United States. On average, the prognosis is less than one year from the time of diagnosis.

The cancer is named from mesothelium, the thin protective lining where the tumor develops. It can appear on the lining of the lungs, stomach, heart or testicles, known respectively as pleural, peritoneal, pericardial and testicular mesothelioma. Each type of mesothelioma in the lung is associated with a unique set of symptoms, but chest or abdominal pain and shortness of breath affect most patients, regardless of their specific diagnosis (*Schuberth PC et al., 2013*; *Niccoli-Asabella A et al.,2013*).

Lung Cancer. Asbestos-related lung cancer is almost indistinguishable from lung cancer caused by another risk factor. The symptoms are the same, regardless of the cause of the cancer. Even though asbestos is only responsible for a small portion of all lung cancer diagnoses, lung cancer is still one of the most fatal related malignancies. Like MM, lung cancer is most common in people who were exposed to large quantities of asbestos for a

prolonged period of time. Asbestos-exposed smokers also have an increased risk of developing small cell or non-small cell lung cancer (*Iwai K. et al., 2000*).

Molecular mechanisms of asbestos toxicity

Current biomedical literature often refers to the relevance of oxidative stress, mediated by reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) production, redox homeostasis imbalance, and cellular redox signaling in physiological as well as pathological conditions asbestos related (Figure 1.3).

The human body is constantly exposed to oxidants generated both endogenously and exogenously (air pollutants, smoke, cigarettes) and aerobic organisms are naturally protected against oxidative damage via enzymatic and non-enzymatic antioxidant systems, able to detoxify reactive intermediates produced in response to this evoked oxidative stress and restore the balance between oxidant and antioxidant species (*Valavanidis, 2013*). When this balance is upset, the occurring alterations or damaged cells may onset as critical components in the pathogenesis of several diseases (*Rahman, 2006*).

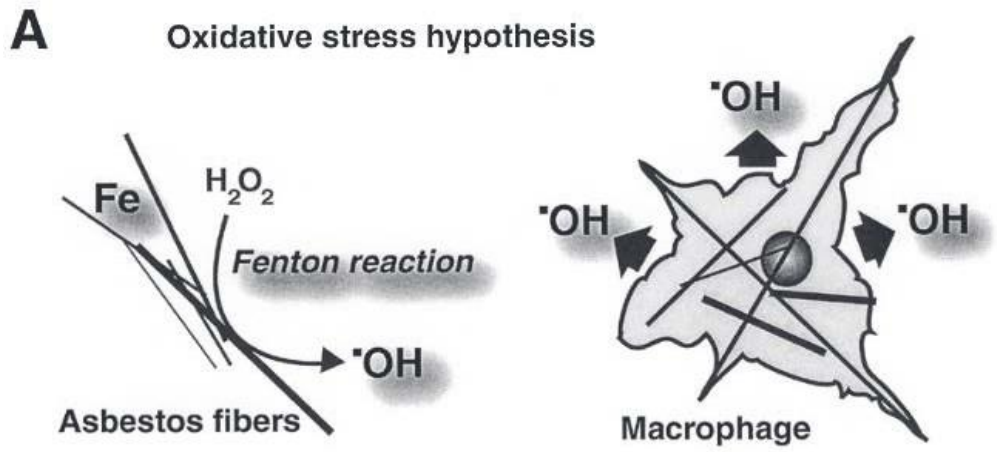


Figure 1.3: Possible mechanism of asbestos induced diseases

One of the mechanism proposed to link these pathological conditions and an evoked oxidative stress is the activation of a very complex process, named **Epithelial-Mesenchymal Transition (EMT)**. Our research group (*Gulino et al., 2016*) showed that chrysotile asbestos is able to induce the secretion of TGFβ from human pulmonary cells (BEAS-2B) and to induce EMT via the activation of a signal transduction path involving Akt/GSK-3β/SNAIl-1. It has been observed the activation of the aforementioned signaling pathway in BEAS-2B is associated with a decrease in epithelial markers (E-Cadherin and β-Catenin) and, on the other hand, an increase in mesenchymal markers (α-SMA, vimentin, fibronectin and matrix metalloproteinases or MMPs). The data obtained through those experiments suggest that chrysotile asbestos induces EMT via a mechanism mediated by TGF.

Epithelial to Mesenchymal Transition (EMT)

The Epithelial to Mesenchymal Transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells. EMT is essential for relevant developmental processes, including mesoderm formation and neural tube formation. EMT has also been shown to occur in wound healing, in organ fibrosis and in the initiation of metastasis for cancer progression (*Tamminen et al., 2012*).

EMT, and its reverse process, MET (Mesenchymal-Epithelial Transition) are critical for development of many tissues and organs (*Thiery JP et al., 2009*). Based on the biological context, EMT has been categorized into 3 types: i) developmental (Type I), ii) fibrosis (*Phua YL. Et al., 2013*) and wound healing (Type II), and iii) cancer (Type III) (*Kalluri et al., 2009*). Loss of E-Cadherin is considered to be a fundamental event in EMT.

Many papers have associated the intracellular generation of ROS with some EMT-triggering mechanisms and signals. *Rhyu et al. (2005)* suggest that TGFβ, NADPH oxidase and the mitochondrial metabolism are important sources of TGFβ–induced cellular ROS, that are in turn responsible for the amplification of the TGFβ signaling in their cellular model. This amplification leads to the activation of Smad2, p38 MAPK, and ERK1/2, a decrease in the E-Cadherin expression and an increase of α-SMA expression and fibronectin secretion, thus supporting the generation of EMT and suggesting that ROS play a key role in TGFβ-induced EMT primarily through activation of MAPK and subsequently through ERK-directed activation of the Smad pathway (*Rhyu et al., 2005*), (Figure 1.4).

Experiments by *Kim et al. (2013)* demonstrated that treatment of human mesothelial cells with H_2O_2, used as a model for excessive ROS production, resulted in altered expression levels of EMT-related genes and that stemness-related genes that may be involved in the survival and aggressiveness of the cancer cells also were significantly increased by the treatment (*Kim, 2013*). In addition, in a recent work *Cichon and Radiski (2014)* show that matrix metalloprotease 3 (MMP-3) is the mediator in inducing EMT, by increasing the expression of the transcription factor SNAIL, through a mechanism that involves the direct binding of the NF-kB subunits p65 and cRel to the promoter of SNAIL.

EMT Markers

As outlined in the previous paragraph, EMT consists of a series of biochemical and morpho-structural events during which a cell loses its epithelial characteristics in favor tothe acquisition of a mesenchymal phenotype. In the tissues, the individual cells that make them are polarized, adhered to a basal lamina and to each other. Such cell-cell junctions and cell-core substance are by means of anchoring proteins, among which, the Cadherines, responsible for cell-cell connections and the integrins, responsible for the cell-core connection.

The most well-known and important among the Cadherines, in the study on EMT, is the **Epithelial Cadherin (E-Cadherin)**. The main function of the E-Cadherin is in the anchoring junctions. Thus, E-Cadherin is the most important epithelial marker in the EMT study (*Wong SHM et al., 2018*). A deficiency of the genes responsible for the expression of E-Cadherin may create a predisposing condition for the onset of epithelial neoplasms, facilitating the metastatic process of the lesion. During an EMT event, this junctions lost and the cell loses its polarity by acquiring migratory capacity, typical of mesenchymal cells. Other proteins involved in maintaining the cellular epithelial phenotype and decreased in EMT are: i) **Claudine**, a family of proteins that are very important components in narrow joints, where they establish a para-cellular barrier, and control the flow of molecules into the intercellular space between the cells of an epithelium; ii) **ZO-1 protein, or Zonula Occludens-1**, also known as Tight junction protein-1 (*Mohandas TK et al., 1995*). It belongs to the family of zonula occludens proteins (ZO-1, ZO-2, and ZO-3), which are tight junction-associated proteins and, among which, ZO-1 is the first to be cloned (*Stevenson BR et al., 1986*).

Conversely, loss of epithelial markers occurs in favor of increased markers of mesenchymal nature, including: i) **Fibronectin**, a high-molecular weight (~440kDa) glycoprotein of the extracellular matrix that binds to membrane-spanning receptorproteins called integrins (*Pankov R. et al., 2002*). Fibronectin exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds(*Pankov R. et al., 2002*). Two types of fibronectin are present in vertebrates: soluble plasma fibronectin (formerly called "cold-insoluble globulin", or CIg) and insoluble cellular fibronectin, the major component of the extracellular matrix (*Pankov R. et al., 2002*).

Fibronectinplaysamajorroleincelladhesion,growth,migration,anddifferentiation,

and altered fibronectin expression, degradation, and organization has been associated with a number of pathologies, including cancer and fibrosis (*Williams CM et al., 2008*);
ii) **Vimentin** is a protein expressed in mesenchymal cells and is the major cytoskeletal component of mesenchymal cells (*Conway J, et al., 2017*), and, because of this, vimentin is one of the EMT marker; iii) **alpha-SMA (α–SMA)**, the human aortic smooth muscle actin or alpha smooth muscle actin, is a protein involved in cell motility, structure and integrity, and is commonly used as a marker of myofibroblast formation (*Nagamoto et al., 2000*). By summing up, during a EMT event, there is a decrease of epithelial markers and an increase of mesenchymal markers (Figure1.4).

Transcription Factors involved in EMT

Many transcription factors (TFs) that can repress E-Cadherin directly or indirectly can be considered as EMT-TFs. TGFβ, SNAI1/SNAIL-1, SNAI2/SNAIL-2 (also known as Slug), ZEB1, ZEB2, E47 and KLF8 (Kruppel-like factor 8) can bind to E-Cadherin promoter and repress its transcription, whereas factors such as TWIST, Goosecoid, E2.2 (also known as TCF4), homeobox protein SIX1 and FOXC2 (fork-head box protein C2) repress E-Cadherin indirectly (*Peinado H et al., 2007;Yang & Weinberg, 2008*). SNAIL and ZEB factors bind to E-box consensus sequences on the promoter region. These EMT-TFs not only directly repress E-Cadherin, but also repress transcriptionally other junctional proteins, including claudins and desmosomes, thus facilitating EMT. Since EMT in cancer progression recaptures EMT in developmental programs, many of the EMT-TFs are involved in promoting metastasis (*Nouri M et al 2014*).

Thus, several signaling pathways (TGFβ, FGF, EGF, HGF, Wnt/beta-catenin and Notch) and hypoxia may induce EMT. Recently, activation of the phosphatidylinositol 3' kinase (PI3K)/AKT axis is emerging as a central feature of EMT. Similarly, Hedgehog, nuclear factor-kappaB and Activating Transcription Factor 2 have been implicated to be involved in EMT (*Vlahopoulos SA et al., 2008*; *Huber MA et al 2004*; *Katoh Y andKatoh M2008*).

The signal transduction pathway concerning TGFβ involves a large number of second messengers:it is able to give rise to EMT via SMAD-dependent or SMAD-independent pathways (*Wong SHM, et al., 2018*).

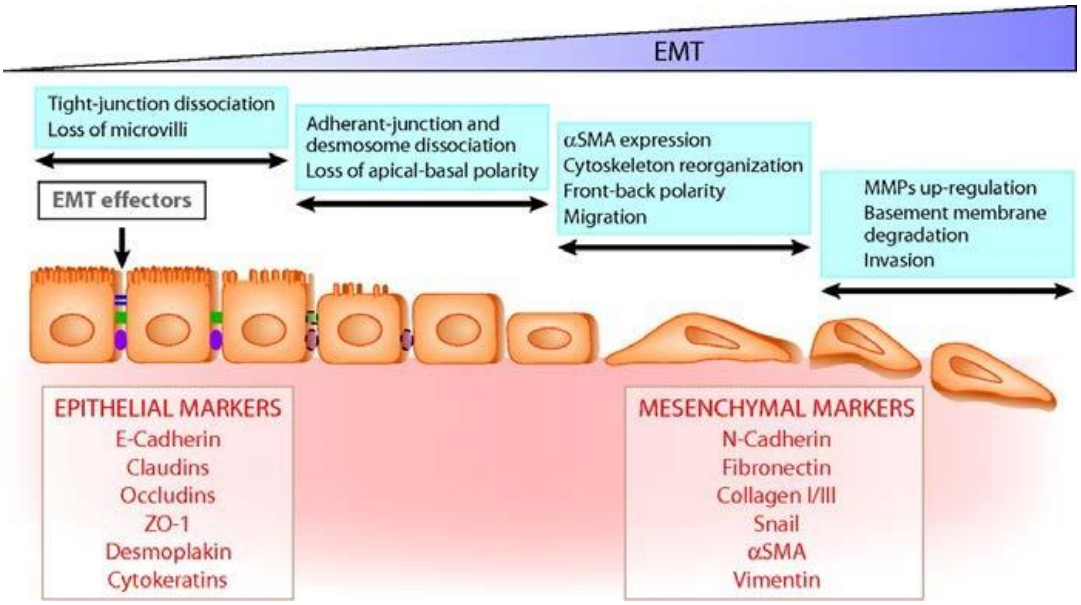


Figure 1.4 Aroeira LS, *et al*. **Epithelial to mesenchymal transition (EMT) and peritoneal membrane failure in peritoneal dialysis patients: pathologic significance and potential therapeutic interventions**. J Am Soc Nephrol. 2007 Jul;18(7):2004-13. http://jasn.asnjournals.org/content/18/7/2004/F2.large.jpg

1.6 TGFβ

The Transforming Growing Factor-beta (TGFβ) is a pleiotropic cytokine that exhibits a broad spectrum of biological and regulatory effects at cellular and organic levels. It plays a key role in growth, in development, differentiation and cell proliferation, in the synthesis and degradation of the extracellular matrix (ECM), in the control of apoptosis and cell cycle progression. Three TGFβ isoforms (1-3) have been described in mammals. They are synthesized by most types of cells and tissues.

TGFβ signal transduction is mediated by TGFβ type I and II receptors, by phosphorylation and changes to receptors conformation via different pathways:

1) Recruitment of SMAD, which enters into the nucleus and interacts with various transcription factors, co-activators and co-repressors. TGFβ induces MAPK- and MAP / ERK kinase-dependent signal transduction pathway and pathway of NF-κB. TGFβ signaling is super-regulated, for example, by interaction with inhibitory SMADs (I-SMADs) or the binding of the enzymes of the ubiquitin-ligase family Smurf1 and Smurf2 and / orco-receptors.
2) In the dependent SMAD pathway, TGFβ ligand binds its own transmembrane receptor, which, immediately after binding, dimers and receptor tyrosine kinase domain leads to phosphorylation of SMAD-2, which, by binding to SMAD-4 , forms a complex that, moving to a nuclear level, interacts with specific target genes involved in changes in the structure of the cytoskeleton and in dissolution of cell joints. Independent SMAD transduction pathways involve molecules such as Rho, Rac and CDC 42, and the PI3K path, which is involved in activating AKT/PKB and mTORC-1. Although there are diverging ways, they are all involved in determining EMT.

Downstream of the transduction pathway, SMAD factors can interact with a spectrum of factors in turn associated with EMT (Figure 1.5). Firstly, SMAD can interact with the family of transcriptional factors SNAIL (*Paznekas WA et al., 1999*) (*Villarejo A. et al., 2014*). About EMT event, SNAIL family promotes the repression of the adhesion molecule E-Cadherin so by inducing EMT during embryonic development.
Another family of transcriptional factors that interacts with SMAD is ZEB (Zinc finger E-box-binding homeobox 1). ZEB1 encodes a zinc finger and homeodomain transcription factor that represses E-Cadherin and T-lymphocyte-specific IL2 gene expression by binding

to a negative regulatory domain (*Williams TM et al., 1991*). ZEB1 and its mammalian paralog ZEB2 belongs to the ZEB family within the ZF (zinc finger) class of homeodomain transcription factors (*Liu W et al., 2014*).

Other downstream transcription factors of SMAD and involved in EMT are: bHLH family, E12 / E47, TWIST and Ids. In particular, the Twist-related protein 1 (TWIST1) is a basic helix-loop-helix transcription factor that in humans is encoded by the TWIST1 gene (*Bourgeois P et al., 1996*)(*Dollfus H et al., 2001*). TWIST is activated by a variety of signal transduction pathways, including Akt, signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinase, Ras, and Wnt signaling. Activated TWIST upregulates N-Cadherin and downregulates E-Cadherin, both hallmarks of EMT. Moreover, Twist plays an important role in some pathological processes like metastasis, angiogenesis, extravasation, and chromosomal instability (*Xie X, et al. 2017*).

TGFβ is over-expressed in a multitude of human pathologies, such as fibrosis and cancer, both related to EMT event.

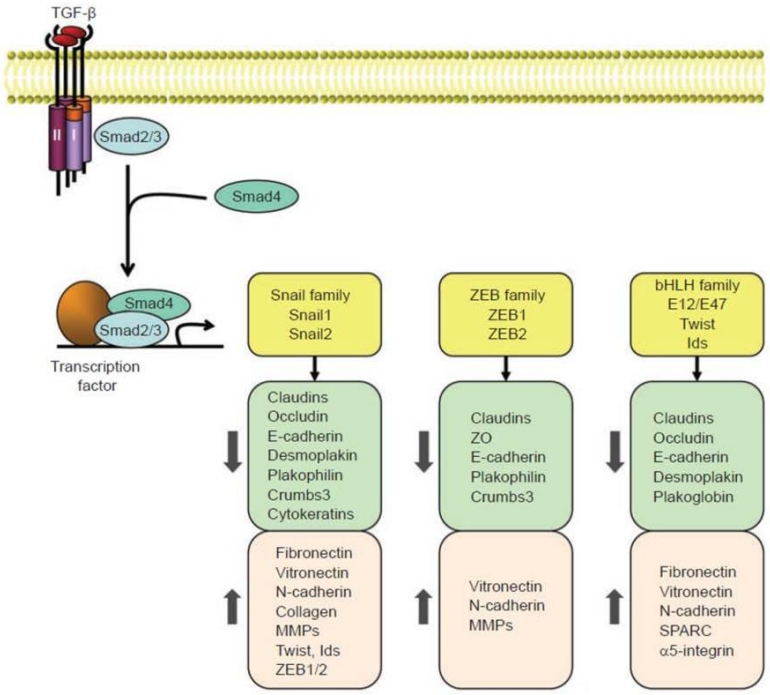


Figure 1.5: Transcriptional factors, resulting from the TGF-β pathway, conjugated to EMT

1.7 Asbestos and EMT

Many studies have investigated the effects of asbestos and its role in EMT. Tamminen et al (2012), showed that asbestos can induce EMT in lung epithelioma A549 cells. In their experiments, they exposed cultured human lung epithelial cells to crocidolite asbestos and analyzed alterations in the expression of epithelial and mesenchymal marker proteins and cell morphology. Asbestos was found to induce downregulation of E-Cadherin A549 lung carcinoma cells (*Tamminen et al., 2012*).A549 cells also exhibited loss of cell-cell contacts, actin reorganization and expression of α-Smooth Muscle Actin (α-SMA).

Asbestos induces lung fibrosis via increased secretion of TGFβ (*Manning et al. 2002*), particularly in idiopathic pulmonary fibrosis, where TGFβ has been localized in association with bronchiolar epithelial cells and their extracellular matrix (*Liu and Brody 2001; Pociask et al. 2004*). *Qi et al. (2013)* compared the toxicity of two different kinds of asbestos: chrysotile and crocidolite. The contact of the fibers with the pulmonary epithelium induces inflammation and oxidative stress, directly correlated with the development of EMT (Figure 1.6) In their work, Qi et al. suggested that continuous exposure to crocidolite and chrysotile could cause EMT of human mesothelial cells via High Mobility Group Box 1 (HMGB1) and TNF-α signaling. In particular, the authors found that repeated exposure to chrysotile and crocidolite led to similar molecular changes and to similar amounts of HMGB1 secretion in vitro and in vivo, with differences in inducing MM-related biological alterations according to their biopersistence (*Qi et al. 2013*). For these reasons, interest in the role of asbestos as an inducer of EMT has recently increased. Given the strong association of chrysotile exposure with TGFβ activation (*Murthy et al. 2015; Pociask et al. 2004*), which is in turn associated with EMT induction, in our research group *Gulino et al., (2016)* investigated the role of chrysotile in inducing EMT via TGFβ in a human bronchial epithelial cellular model (BEAS-2B), to increase knowledge of the molecular bases of asbestos-related lung diseases. In their work, they concluded that chrysotile is able to trigger EMT in BEAS-2B cells through a molecular mechanism involving TGFβ and its intracellular effectors Akt/GSK-3β/SNAIL-1. Their findings suggest just one of the possible molecular mechanisms supporting the morphological transformations typical of EMT involved in asbestos-related disease such as

asbestosis. Therefore, it is conceivable that additional mechanisms will be unveiled in the future.

Different studies have explored the role of EMT in MM, the worst disease correlated to asbestos exposure. *Casarsa et al. (2011)* expressed the importance of EMT markers in MM prognosis. Few studies have evaluated the prognostic value of EMT markers in MM (*Schramm A. et al., 2010*; *Casarsa C. et al., 2011*). *Iwanami et al., (2013)* hypothesized that the mesenchymal characteristics might predominate in the epithelioid-type tumors, and that the deregulated EMT status may indicate aggressive behavior in MM. If this is the case, detection of the EMT status might allow for the identification of patients at a high risk for disease progression. Therefore, authors found that positive expression of EMT-related molecules was more common in patients with MM.

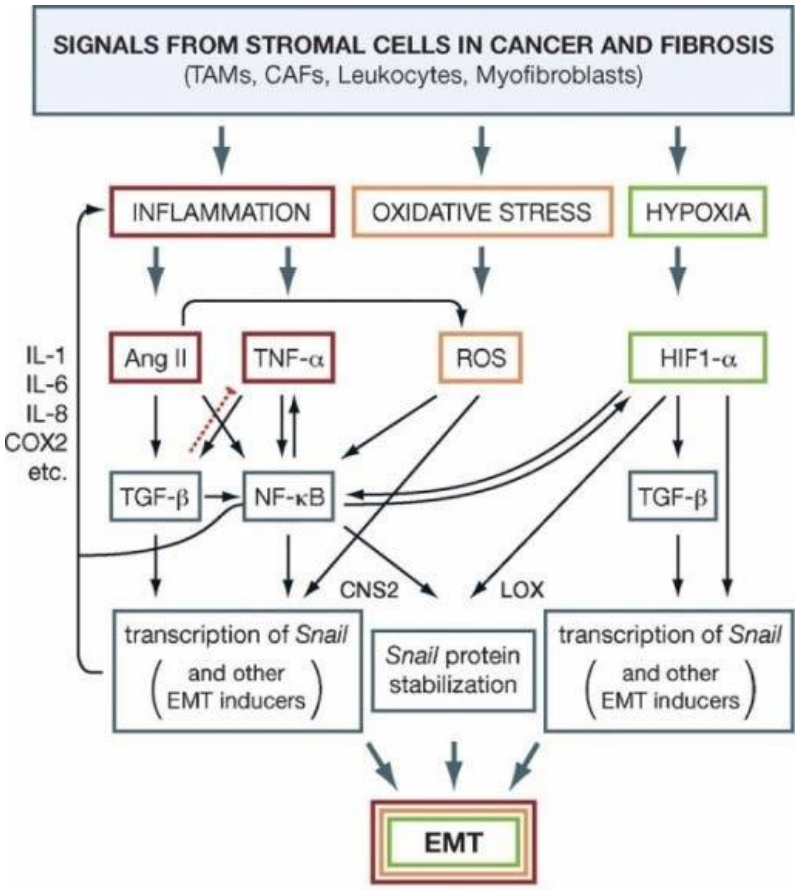


Figure 1.6 Inflammation, oxidative stress and hypoxia cooperate in the induction of EMT for the progression of organ fibrosis and cancer metastasis

2. Aim of work

The aim of this PhD thesis is to study the mechanism by which chrysotile asbestos is able to induce EMT in human mesothelial cells (MeT-5A). Goals to reach:

1. if human mesothelial cells MeT-5A, exposed to chrysotile asbestos, have EMT and if this effect may be mediated by TGFβ;
2. if TGFβ is secreted after asbestos exposure, and if this event mediates EMT by downregulation of E-cadherin, via SMAD/SNAIL factors(GSK-3β);
3. if ZEB and TWIST as two others possible SMAD-dependent TGFβ mediators in EMT induced by asbestos fibres.

3. Materials and Methods

Cells and Reagents

Human mesothelial cells (MeT-5A) cells were obtained from American Type Culture Collection (ATCC® CRL-9444™, ATCC, Manassas, VA).The above cells are not tumor cells and have been immortalized by transfection with pT RVS plasmid. Cells were cultured in Medium199 (M199) (Gibco, Paisley, UK) supplemented with 10% FBS and 1% penicillin/streptomycin, without glutamine, in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

The protein content of the monolayers and cell lysates was assessed with the BCA kit from Pierce (Rockford, IL). Plastic ware was purchased from Falcon (Becton Dickinson, Franklin Lakes, NJ). Unless otherwise specified, other reagents were purchased from Sigma-Aldrich. For all experiments, ultrapure water (Millipore, Bedford, MA) was used.

Experimental Conditions

Asbestos

To assess the appropriate concentration and time for the incubation of MeT-5A cells with Chrysotile asbestos fibres (Chrysotile UICC, Union Internationale Contre le Cancer) dose and time-dependence experiments were performed (data not shown). As a consequence of these preliminary results, we chose to seed 4.5 x 10^5 cells in 100 mm-diameter Petri dishes and incubate them for 72 and 96 h in the absence or presence of 5 µg/$cm^2$of Chrysotile asbestos suspension in M199medium. At the above-mentioned asbestos concentration, a high cellular mortality was shown, confirmed by the LDH test (data not shown).

Asbestos samples

To promote bundle separation and obtain specimens suitable for biological tests, asbestos sample have been suspended in Medium M199 and sonicated for t < 1 min at 100 W/ml and 20 kHz with a probe sonicator (Labsonic Sonicator; Sartorius Stedim Biotech S.A., Aubagne, France).

TGFβ Solution

A solution of recombinant TGFβ was obtained by resuspending the lyophilized form of hrTGFβ, Human Recombinant TGFβ, (PeproTech, Rocky Hill, USA) in a 10 mM citric acid solution, with a pH of 3.0 and, in order to increase the storage time. The previously prepared solution was diluted in a 0.1% BSA based buffer. The final concentration of TGFβ solution, before incubation in the dish, is 10 ng/μl.

Cellular Tests

Experimental conditions.

Human mesothelial cell line (Met-5A) were incubated with a chrysotile suspension (in M199 medium supplemented with FBS 10% and Penicillin/Streptomicin 1%) at the concentration of 5 $\mu g/cm^2$ (after a dose-dependent test, data not shown) and a TGFβ solution at the concentration of 10 ng/μl for 72-96 hours incubation.

Western Blot Analysis

Cell lysates were obtained by dissolving cells in sample buffer 2X (25 mM Tris/HCl, pH 6.8, 10% SDS, 10% Glycerol), supplemented with the protease inhibitor cocktail set III (100 mM AEBSF, 80 μM Aprotinin, 5 mM Bestatin, 1.5 mM E-64, 2 mM Leupeptin and 1 mM epstatin; Calbiochem-Novabiochem Corporation, La Jolla, CA). Nuclear extracts were obtained using the Active Motif nuclear extraction kit (Active Motif, Rixensart, Belgium), according to the manufacturer's instructions. Antibodies against E-Cadherin, Fibronectin, β-Catenin, Tubulin and SNAIL-1 were all provided by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-Vimentin antibody was provided by Sigma Chemical Co. The anti-α-SMA (Smooth Muscle Actin) antibody was provided from GeneTex (Irving, CA).Cell lysates and nuclear extracts were separated by SDS-PAGE, transferred to PVDF membrane sheets (Immobilon-P, Millipore) and probed with the required antibody diluted in PBS-Tween 0.1% with Blocker Non-Fat Dry Milk 1%. After 1 h of incubation the membranes were washed with PBS-Tween 0.1% and then subjected for 1 h to a peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (from sheep; Amersham International, Bucks, UK),

diluted1:2000inPBS-Tween0.1%withBlockerNon-FatDryMilk1%.Themembraneswere washed again with PBS-Tween 0.1% and proteins were detected by enhanced chemiluminescence (Perkin Elmer, Shelton, CT).

TGFβ detection

In order to determine the possible release of TGFβ into the culture medium, the MeT-5A cells were incubated with the above mentioned conditions and subsequently the supernatants were collected and tested, by ELISA method, using a TGFβ Elisa Kit provided by Invitrogen Corporation (Flynn Road, Camarillo, California, USA). The immunoenzymatic assay was performed in accordance with the indications on the bug, annexed together with the kit. Of each culture medium sample (untreated cells sample, Chrysotile-treated cells sample) were taken 250 μl and added to 50 μl of Extraction Solution (provided in the kit). Subsequently, they were incubated for 30 'to 4°C and added to 250 μl of Standard Diluent Buffer (supplied in kit), according to manufacturer's instructions. The determination of TGFβ concentration is done according to a calibration curve, made from various TGFβ standards. A TGFβ solution is obtained at a concentration of 10.000 pg/ml. The prepareddilutionshadthefollowingconcentrations:2000, 1000, 500, 250, 125, 62.5 and 31.2 pg/ml of TGFβ1. The method also provided for diluting the Streptavidin-HRP Working Solution solutions and Washing Solution or Wash Buffer, both supplied in the kit, according to the indications in the bug. The method continued with the preparation of the wells, which were also supplied in the kit on the bottom of which the primary anti- TGFβ antibody was immobilized. They are added sequentially with 200 μl of Standard Diluent Buffer And 200 μl of standard samples of the curve and unknown samples. Next, 50 μl of secondary antibody solution, biotin-conjugated anti-TGFβ-biotin (Biotin Conjugate) solution, is also supplied by the kit, then covering the plate for 3 hours. There are sequentially four washings performed with diluted Wash Solution, and the addition of 100 μl Streptavidin-HRP Working Solution. The plate is covered and incubated for 30 minutes at room temperature, followed by four further washings, performed with diluted Wash Solution, and the addition of 100 μl of Stabilized Chromogen. It covers the plate, incubating it again for 30 minutes at room temperature, and finally, 100 μl of Stop Solution, supplied in the kit, are added. The last step is to read the plate, at the microplate, to a λ(wavelenght) of 450 nm, returning the data obtained on a spreadsheet in Excel.

The neutralizing anti-TGF-β antibody was purchased by Abcam (Abcam, Cambridge, United Kingdom) and was used at a concentration of 5 μg/ml.

qRT-PCR

Total RNA was obtained by the guanidinium thiocyanate-phenol-chloroform method.
0.2 μg of total RNA were reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories AG, Glattbrugg, Switzerland) according to the manufacturer's instructions. qRT-PCR was carried out using IQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. PCR amplification was: 1 cycle of denaturation at 94°C for 3 min, 45 cycles of denaturation at 94°C for 30 s, annealing for 30 s, and synthesis at 72°C for 30 s. The relative expression of each target gene was performed comparing each PCR gene product with the S14 ribosomal subunit product using the Gene Expression Macro (http://www3.biorad.com/LifeScience/jobs/2004/04-0684/genex.xls;Bio-Rad).

Gelatin Zymography

Since fetal bovine serum contains Matrix Metallo Proteases (MMPs), cells were cultured in 1% serum medium only. Afterwards, culture supernatants were collected, supplemented with Laemmli sample buffer (Tris HCl 0,5 M pH 6.8, SDS 10%, Glycerol 30%, 15% β–Mercaptoethanol, 0,03% Bromofenol Blue)and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 1 mg/ml gelatin (separating gel 10%), under non-denaturing and non-reducing conditions. After electrophoresis, gels were washed at room temperature for 2 h in milliQ water containing 2.5% (v/v) Triton X-100 and incubated for 18 h at 37°C in a Collagenase Buffer containing the following: 200 mM NaCl, 50 mM Tris, 10 mM $CaCl_2$, and 0.018% (v/v) Brij 35 (pH 7.5) with or without 5 mM EDTA to exclude unspecific bands. At the end of the incubation, gels were stained for 15 min with Coomassie blue (0.5% Coomassie blue in 30:10:60 methanol/acetic acid/water). Gels were destained in 75% methanol/25% glacial acetic acid.

Statistical Analysis

The results were analyzed by a one-way Analysis of Variance (ANOVA) and Tukey's test (software: SPSS 11.0 for Windows, SPSS Inc., Chicago, IL). A $\rho<0.05$ was considered significant.

4. Results

Chrysotile asbestos induces EMT in MeT-5ACells

MeT-5A cells were incubated as described in Materials and Methods. After incubation with chrysotile fibres (CTL) or TGFβ, cells acquired a characteristic fibroblastoid morphology (Figure 4.1-4.2) and appeared more elongated and thinned in spite of untreated cells (CTRL), typical of EMT event.

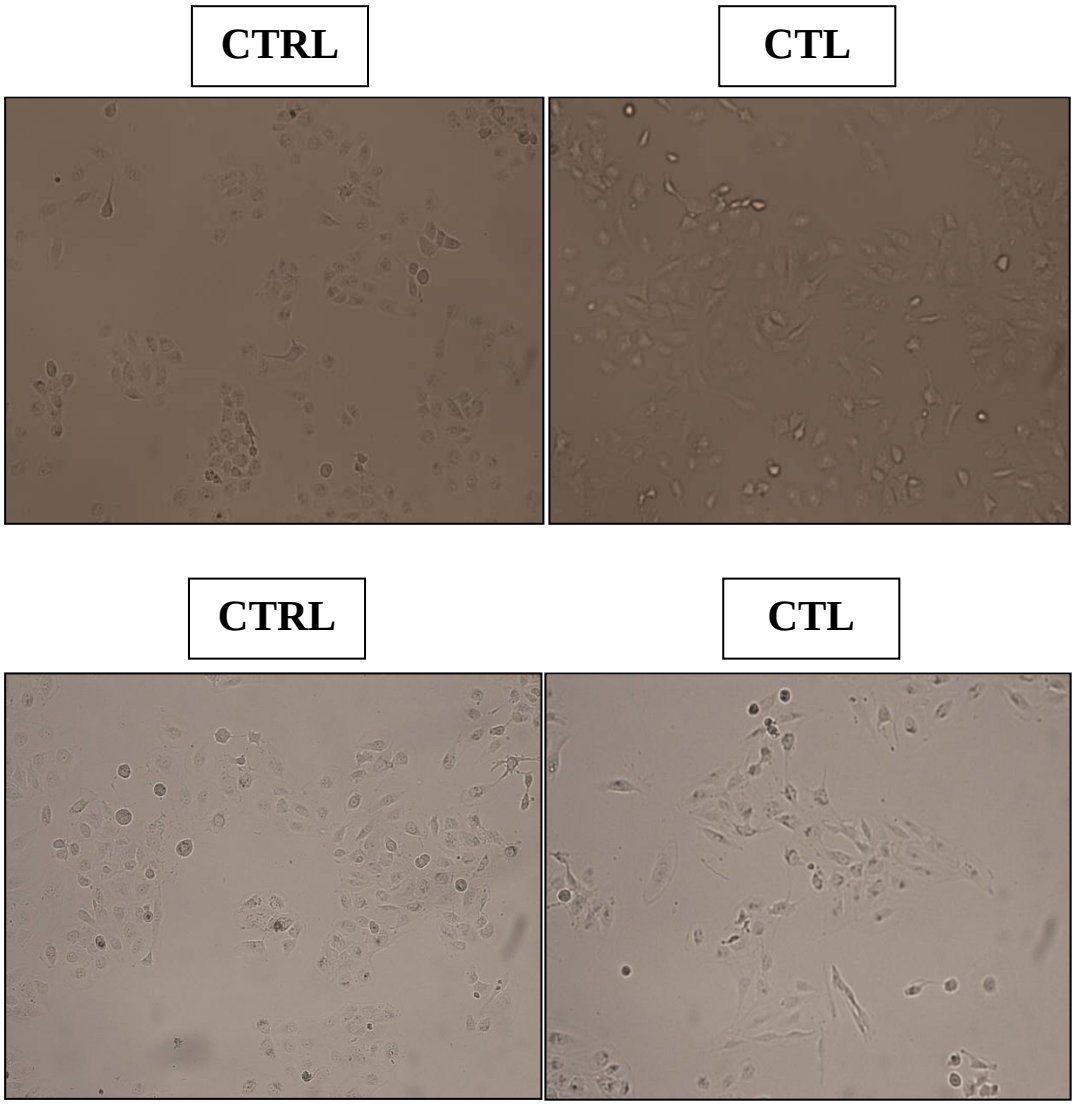


Figure 4.1: Morphological changes induced by chrysotile asbestos fibers. MeT-5A cells were cultured in the absence (CTRL) or presence (CTL) of 5 µg/cm^2 of chrysotile asbestos fibres suspension for 72 and 96 h of incubation. MeT-5A cells changed their morphology from the typical shape of untreated cells to a spindle-shaped fibroblast-like morphology. The white arrows highlight the cellular elements of which there is a change in morphology.

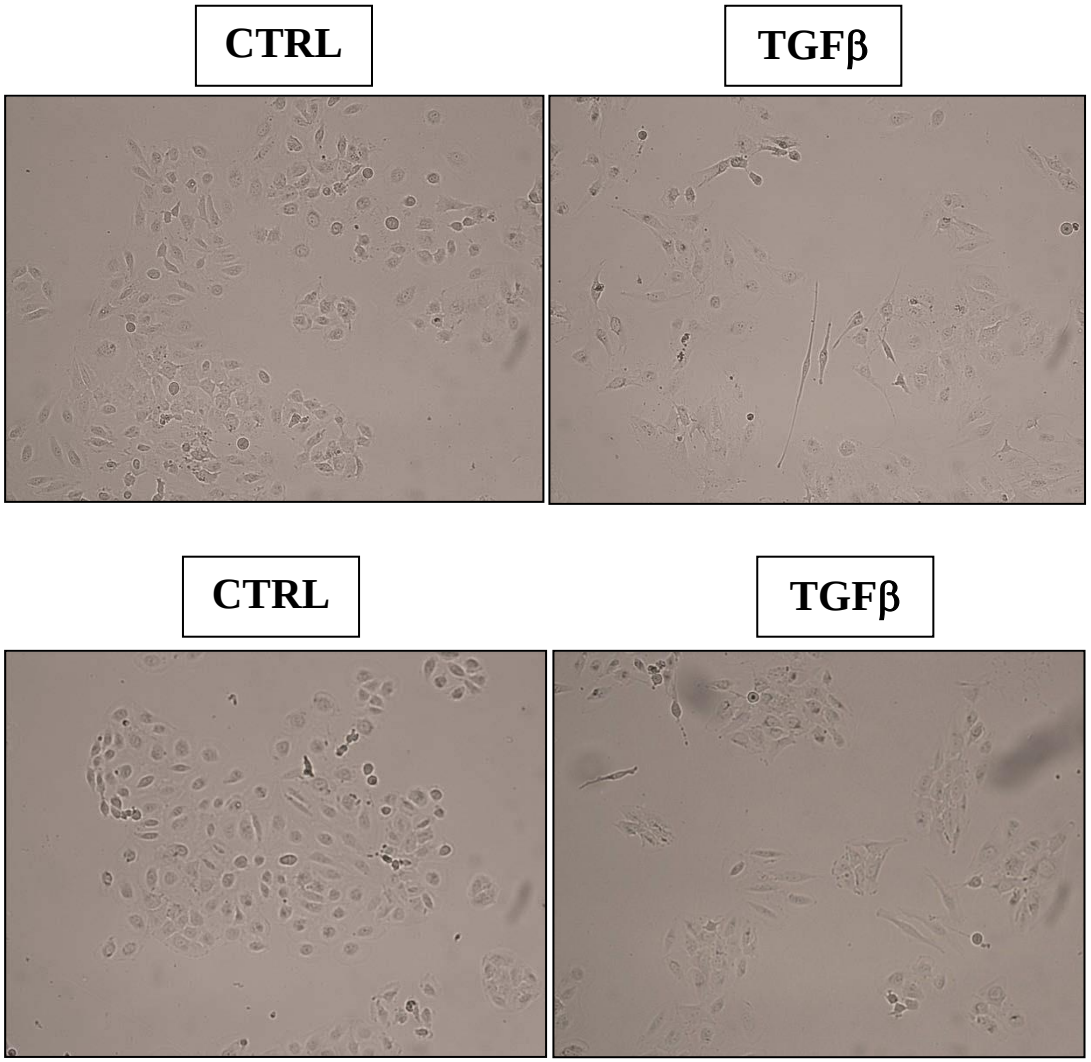
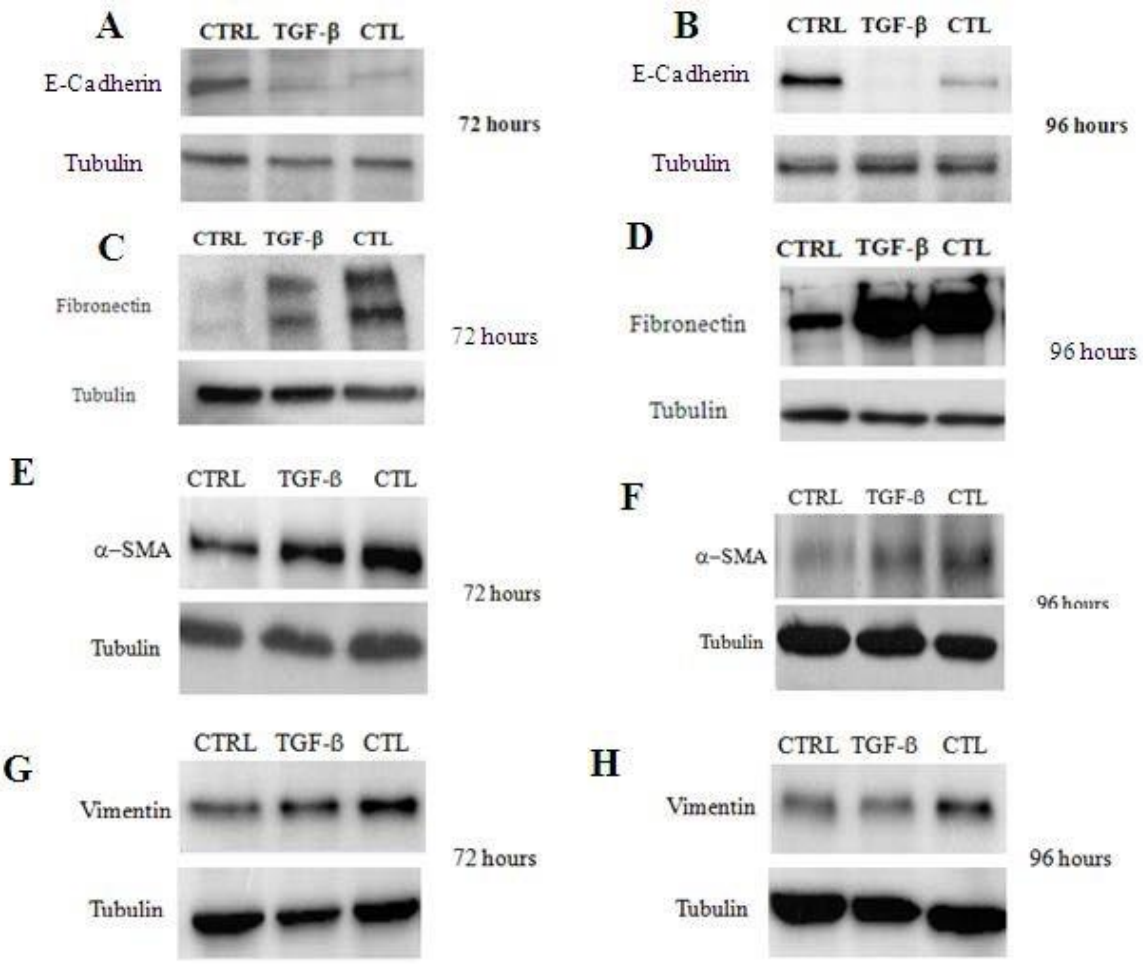
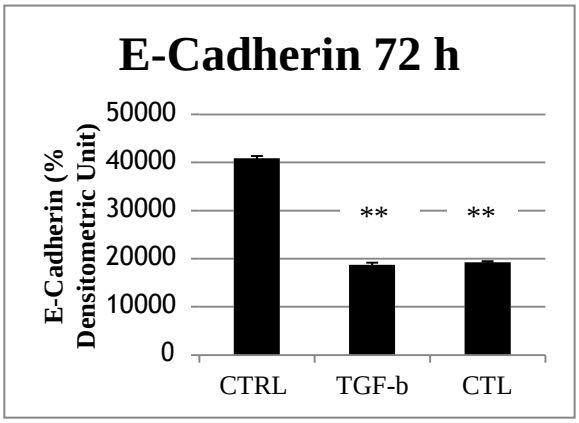


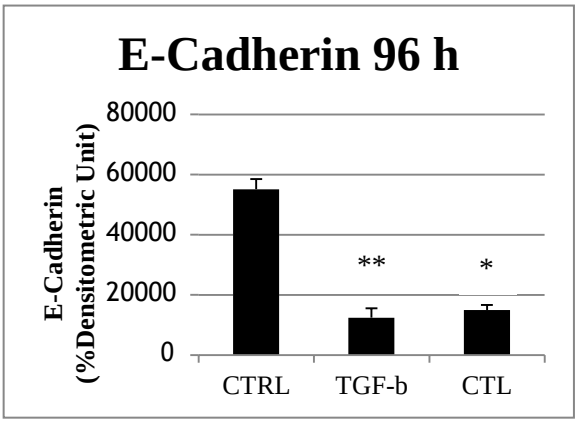
Figure 4.2: Morphological changes induced by TGFβ. MeT-5A cells were cultured in the absence (CTRL) or presence (TGFβ) of 10 ng/µl of TGFβ solution for 72 and96 h of incubation. MeT-5A cells changed their morphology from the typical shape of the control cells to a spindle-shaped fibroblast-like morphology. The white arrows highlight the cellular elements of which there is a change in morphology.

Chrysotile downregulates epithelial markers and upregulates mesenchymal markers in MeT-5A Cells

MeT-5A cells exposed to CTL showed a significant decrease in the level of the epithelial marker E-Cadherin, the main epithelial marker in EMT, unlike untreated cells (Figure 4.3 A-B). In parallel, it was observed an increase of the mesenchymal markers as Fibronectin, Vimentin and α–SMA after asbestos incubation, unlike untreated cells (Figure 4.3 C-D-E-F-G-H).




E-Cadherin 72 h
E-Cadherin (% Densitometric Unit)
50000
40000
30000
20000
10000
0
**
**
CTRL
TGF-b
CTL


E-Cadherin 96 h
E-Cadherin (%Densitometric Unit)
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60000
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0
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*
CTRL
TGF-b
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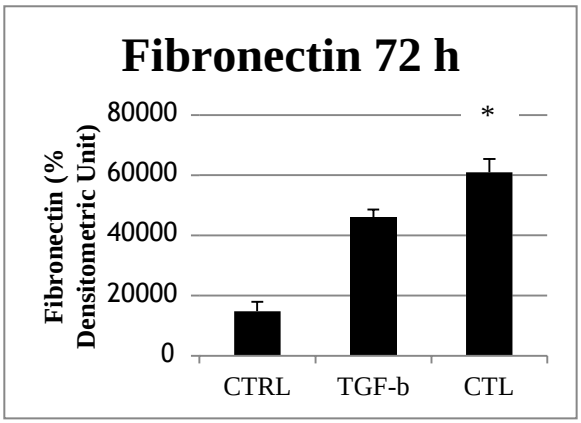
Fibronectin 72 h
Fibronectin (% Densitometric Unit)
80000
60000
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20000
0
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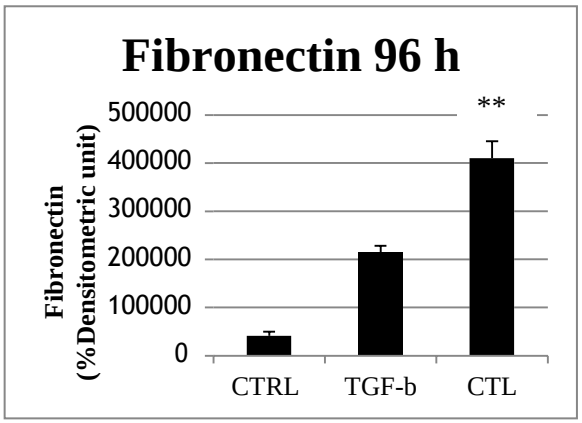
TGF-b
CTL


Fibronectin 96 h
Fibronectin (%Densitometric unit)
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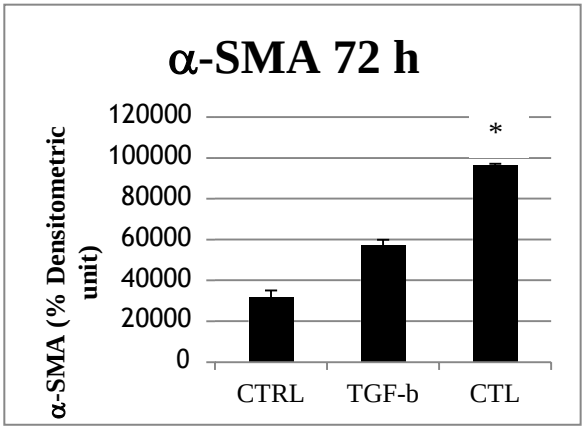
400000
300000
200000
100000
0
**
CTRL
TGF-b
CTL


α-SMA 72 h
α-SMA (% Densitometric unit)
120000
100000
80000
60000
40000
20000
0
*
CTRL
TGF-b
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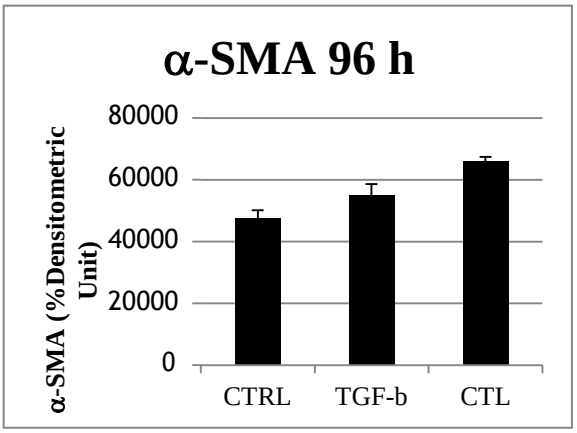
α-SMA 96 h
α-SMA (%Densitometric Unit)
80000
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0
CTRL
TGF-b
CTL

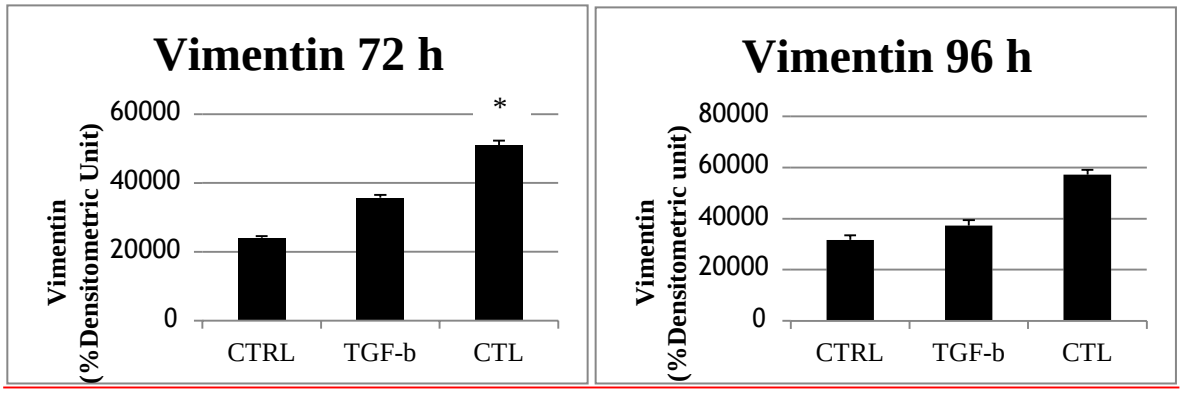
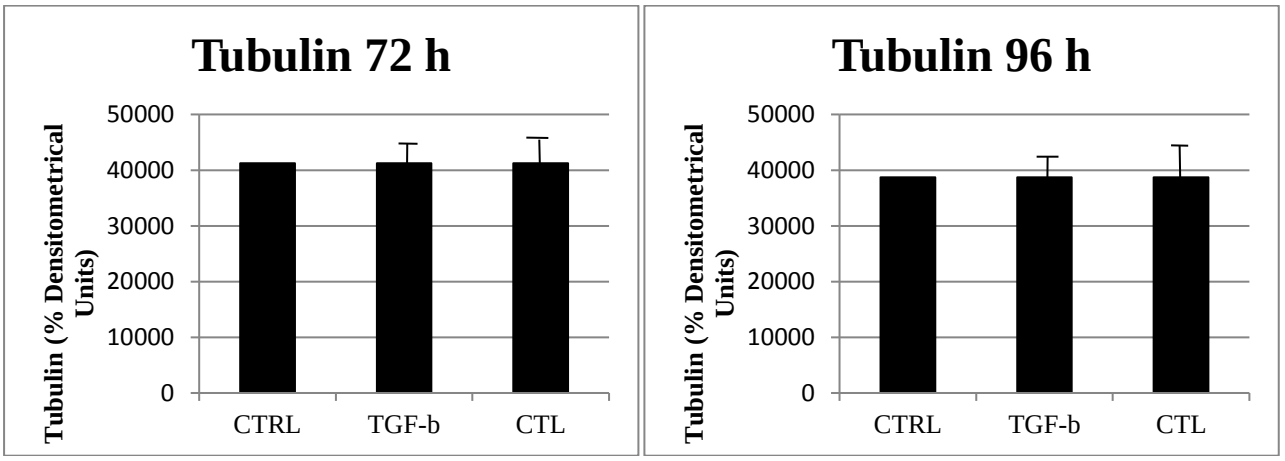


Figure 4.3: **Chrysotile asbestos induces alterations in proteins involved in EMT**. The Western Blotting revealed that the levels of the typical epithelial marker E-Cadherin was significantly decreased in MeT-5A cells treated for 72 and 96 h with 5 µg/cm^2 chrysotile asbestos fibers (CTL), while the mesenchymal proteins α-SMA, Vimentin and Fibronectin were over-expressed in the same conditions, compared to the control. Measurements were performed in triplicateanddataarepresentedasmeans±SEM(n=3).Significancevsrespectivecontrol:*ρ<0.02;**ρ<0.001; ***ρ<0.0001.



Both at 72 and 96 hours of incubation, chrysotile and TGFβ treatment resulted in a statistically decrease in the relative expression of E-Cadherin's mRNA. Regarding the Fibronectin marker, statistically significant increases were observed at 72 and not at 96 hours of incubation with chrysotile or TGFβ(Figure 4.4). Similarly to Fibronectin, α-SMA marker also showed an increase in the relative expression of the mRNA, in particular at 96 hours of incubation. As for the Vimentin marker, there is a greater increase in the relative expression of the mRNA of the same, in particular at 72 h incubation (Figure 4.4).

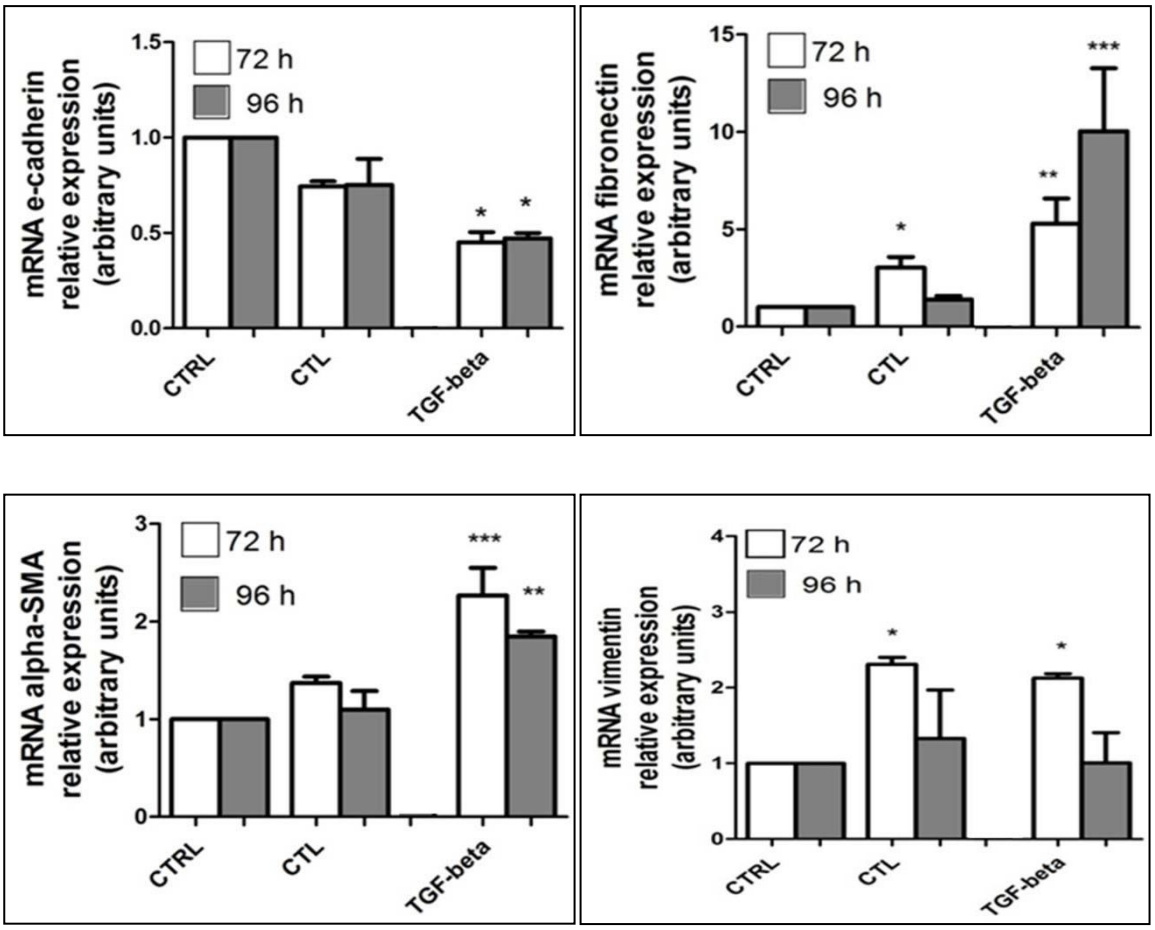
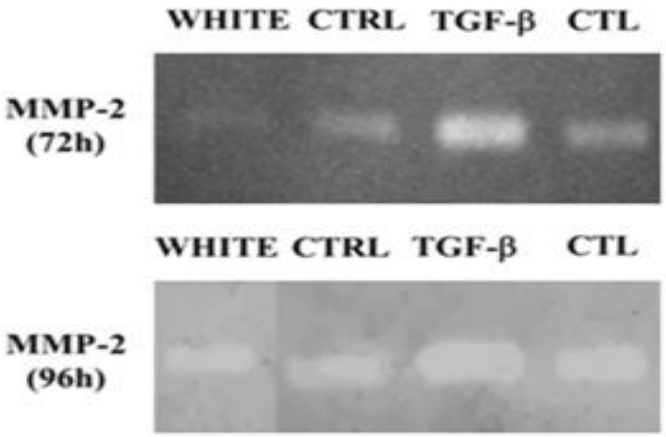
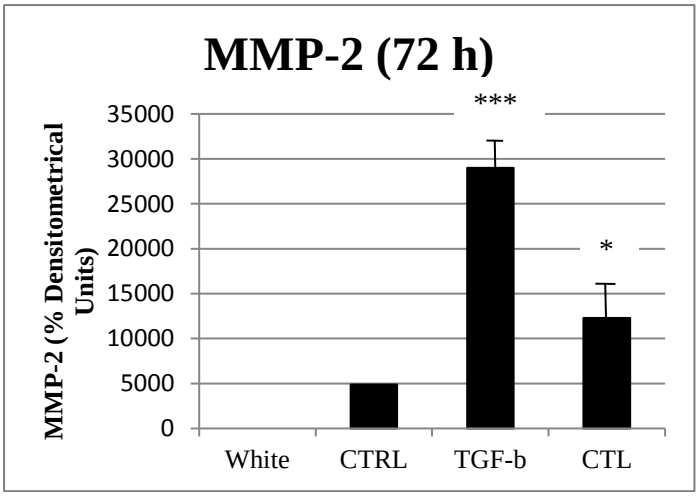


Figure 4.4: qRT-PCR gene expression of selected EMT markers. MeT-5A cells were cultured for 72 h and 96 h without (CTRL) or with 5 μg/cm^{2}of chrysotile asbestos fibers (CTL), and 10 ng/μl of TGFβ. Total RNA was extracted, reverse-transcribed and amplified by RT-PCR to check the relative gene expression of the proteins E-Cadherin, Fibronectin, α-SMA, Vimentin. Measurements were performed in triplicate and data are presented as means ± SEM (n = 3). Significance *vs* respective control: *ρ<0.02; **ρ<0.001; ***ρ<0.0001.

Chrysotile increases MMP-2 secretion while EMT event was induced by.

Since MMPs play a key role in the remodeling of the extracellular matrix and appear to be markers of EMT, we investigated their secretion and their activities. In fact, MeT-5A cells exposed to chrysotile or TGFβ excreted more MMP-2 compared with untreated cells (Figure 4.5).The secretion of matrix metalloproteinases was assayed in the cellular supernatant, ie the M199 medium.





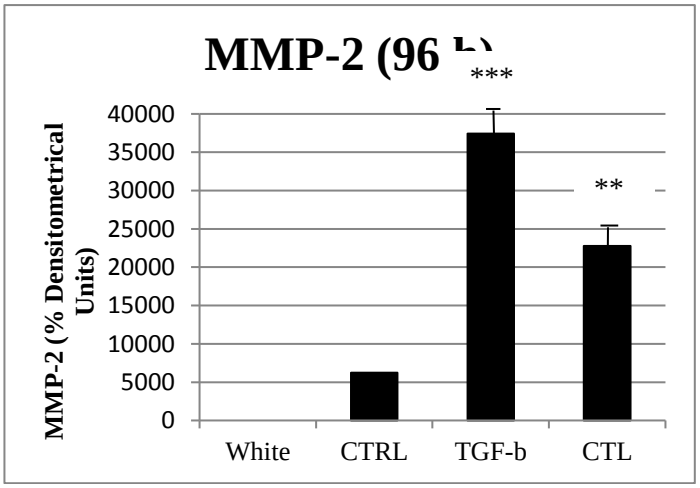


Figure 4.5: Secretion and activity of MMP-2 after incubation with chrysotile asbestos (CTL) and TGFβ solution. MeT-5A cells were incubated without (CTRL) or with 5 µg/cm^2 chrysotile asbestos fibres (CTL) and 10 ng/µl of TGFβ for 72 and 96 h. At the end of the incubation, the levels of MMP-2 were measured in the cell supernatants after normalization. Measurements were performed in triplicate and data are presented as means ± SEM (n = 3). Significance *vs* respective control: *ρ<0.02; **ρ<0.001;***ρ<0.0001.

Exposure to chrysotile asbestos increases TGFβ secretion in MeT-5A cells, and the co-incubation with TGFβ antibody partially restores the basal expression level of EMT markers.

Chrysotile asbestos exposure has already been associated with the increased activation of TGFβ pathway (*Liu and Brody 2001; Manning et al. 2002*): ROS generated by the iron contained in asbestos mediate the biological activity of TGFβ (*Pociask et al. 2004*).

TGFβ levels were measured in MeT-5A cells esposed to chrysotile asbestos. Results showed TGFβ secretion significantly was increased (Figure 4.6).The results show that there is no temporal dependence and that the values of secretion of TGF-b are comparable for 72 and 96 h. A neutralizing anti-TGFβ antibody was used to confirm TGFβ as the mediator of the reported protein changes. As shown in figure 4.7, E-Cadherin was significantly decreased and Vimentin and Fibronectin significantly increased in cells treated with chrysotile asbestos (CTL), while the co-incubation of cells with TGFβ blocking antibody partially restored the protein expression levels (Figure 4.7).

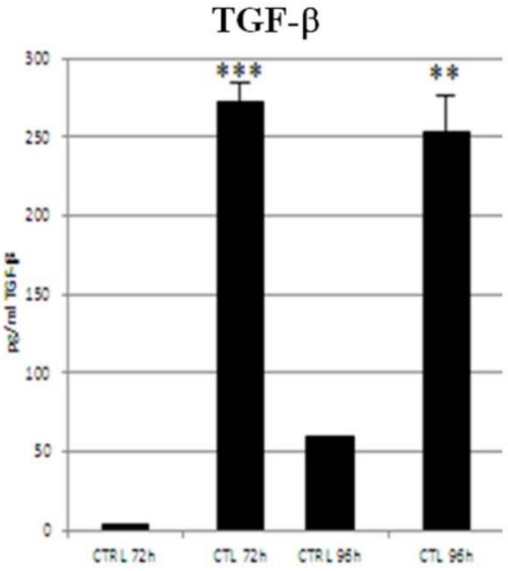
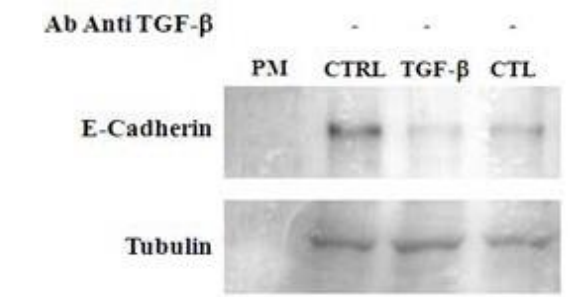
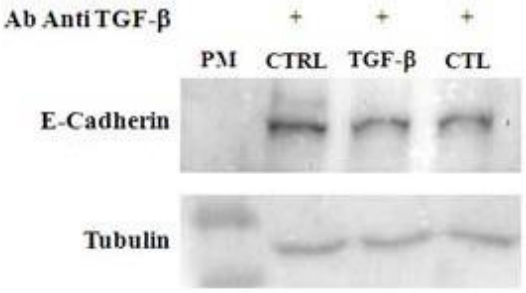
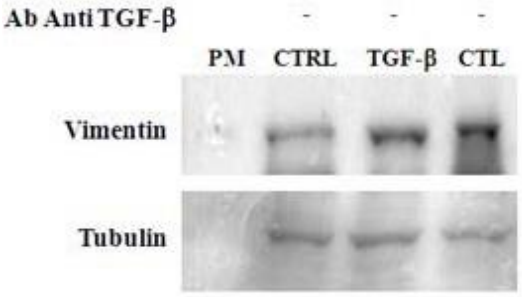
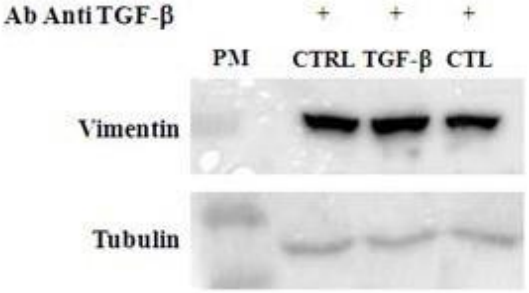


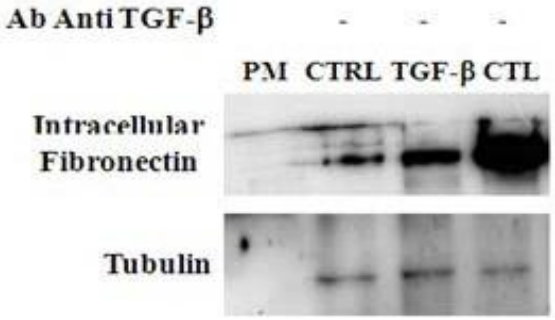
Figure 4.6: **Chrysotile asbestos induces TGF-β secretion.** MeT-5A cells were incubated in the absence (CTRL) or presence (CTL) of 5 μg/cm^2 chrysotile asbestos for 72 and 96 h. At the end of the incubation, supernatants were collected and TGFβ levels were detected through an ELISA kit. Data are shown as means ± SEM (n = 3). TGFβ levels are reported as pg/mg of intracellular proteins. Based on one of the assumptions outlined in the Purpose of Work section, namely that Chrysotile induces TGFβ secretion, strong EMT inductor, I performed a dosage, by E.L.I.S.A. of said cytokine, on two sets of MeT-5A cell supernatants harvested after incubation of 72 and 96 hours. As can be seen from the chart, in the sample treated with CTL, the increase in the release of TGFβ˙ expressed in pg/ml, is fairly net compared to the control sample. Based on the results obtained we can state that asbestos Chrysotile induce release of TGF-β in MeT-5A cells. Significance *vs* respective CTRL: **ρ<0,001***ρ<0,0001.

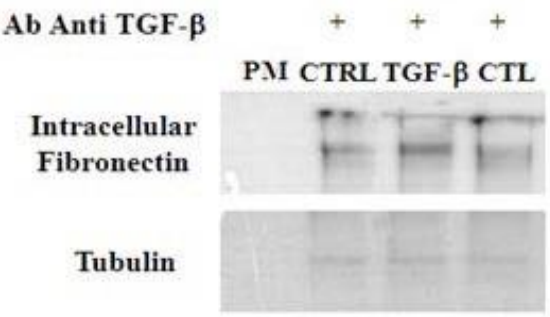

Ab Anti TGF-β - - -
PM CTRL TGF-β CTL
E-Cadherin
Tubulin


Ab Anti TGF-β + + +
PM CTRL TGF-β CTL
E-Cadherin
Tubulin


Ab Anti TGF-β - - -
PM CTRL TGF-β CTL
Vimentin
Tubulin


Ab Anti TGF-β + + +
PM CTRL TGF-β CTL
Vimentin
Tubulin


Ab Anti TGF-β - - -
PM CTRL TGF-β CTL
Intracellular Fibronectin
Tubulin


Ab Anti TGF-β + + +
PM CTRL TGF-β CTL
Intracellular Fibronectin
Tubulin

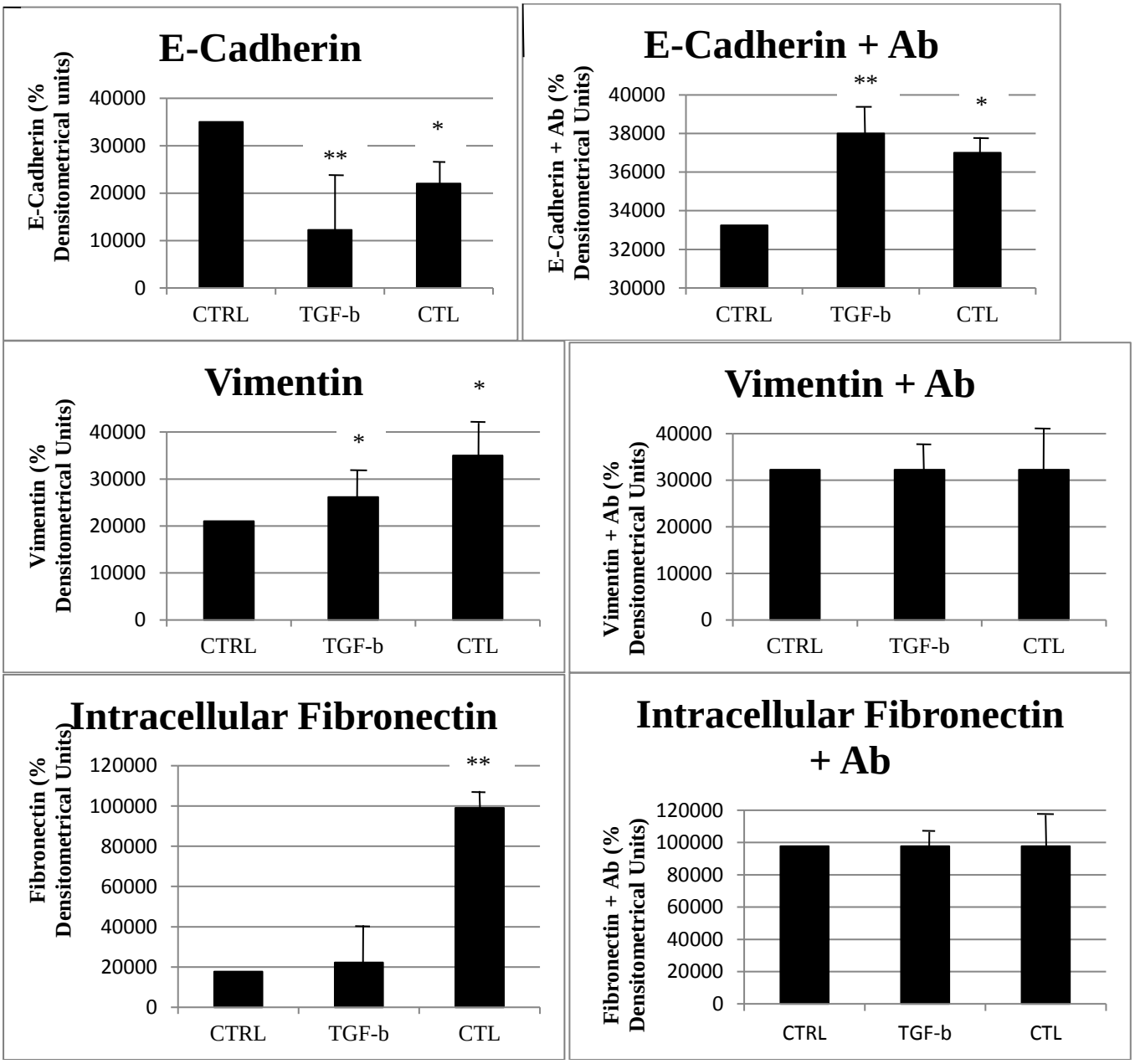
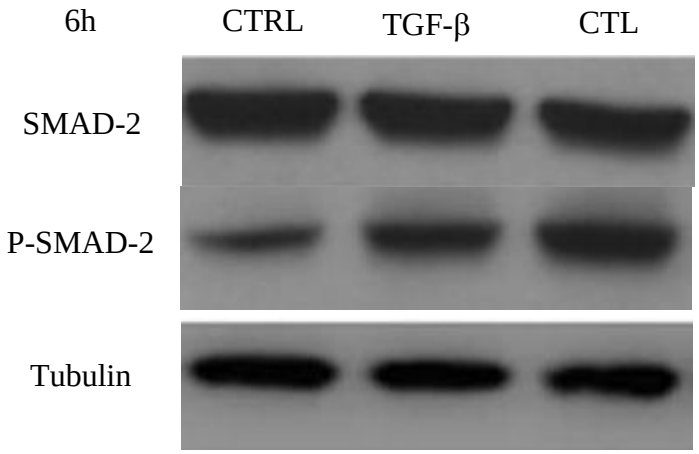


Figure 4.7: The image above shows the behavior of E-Cadherin, Vimentin and Fibronectin, in the presence or absence of anti-TGFβ antibody. In the absence of antibody, E-Cadherin decreased and Vimentin and Fibronectin increased, when cells were incubated TGFβ or chrysotile asbestos fibres. On the contrary, in the presence of the blocking antibody, all markers resulted partially restored. Significance *vs* respective CTRL: **ρ<0,001***ρ<0,0001.

Chrysotile induces SMAD overexpression

As shown above, chrysotile asbestos increases the secretion of TGFβ from MeT-5A cells. TGFβ is able to induce EMT via SMAD dependent or SMAD independent signaling pathway. In the case of a SMAD dependent signalling pathway, TGFβ bind to a type II receptor, which recruits and phosphorylates a type I receptor. The type I receptor then phosphorylates receptor-regulated SMADs (R-SMADs) which can now bind the coSMAD SMAD4. R-SMAD/coSMAD complexes accumulate in the nucleus where they act as transcription factors and participate in the regulation of target gene expression.



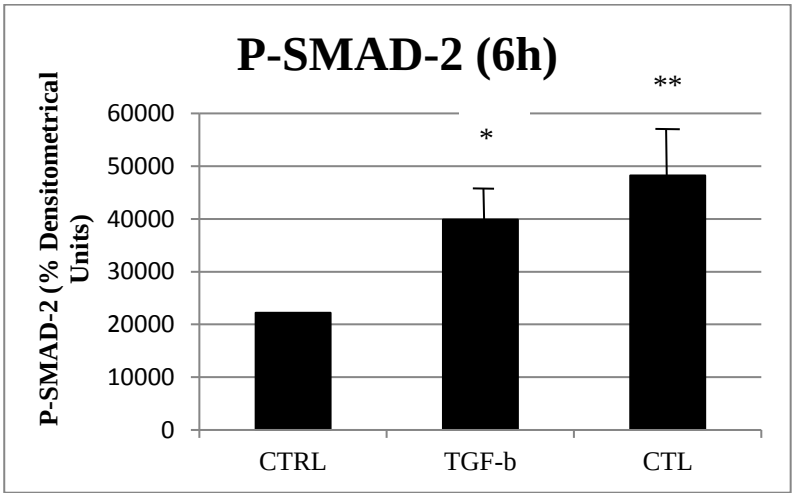


Figure 4.8: Western Blot Analysis of SMAD-2 and P-SMAD-2 factors, in MeT-5A untreated and treated with TGFβ solution (10 ng/μl) and CTL suspension (5 μg/cm^2). Significance *vs* respective CTRL: **ρ<0,001***ρ<0,0001.

The data shown here refers to the Western Blot analysis conducted on SMAD-2 and its phosphorylated active analog P-SMAD-2. In our experimental model, we performed time-dependence experiments (data not shown). A 6 h exposure to chrysotile asbestos or TGFβ resulted in SMAD-2 increased phosphorilation and its accumulation in the nucleus (Figure 4.8) in comparison with untreated cells. On the basis of the results obtained chrysotile, *via* TGFβ increased secretion, is thus able to trigger a SMAD dependent pathway.

Exposure to chrysotile induces E-Cadherin down-regulation through a mechanism mediated by Akt/GSK-3β/SNAIL-1 via the increased secretion of TGFβ.

To investigate the mechanism leading to the down-regulation of E-Cadherin by chrysotile asbestos, we analyzed the involvement of the transcription factor SNAIL-1, one of downstream effector of SMAD dependent pathway driven by TGFβ. The SMAD route involves transcription factors such as SNAIL-1, Twist or ZEB-1, all inducing the E-Cadherin downregulation and, consequently, the start of EMT (*Montserrat N. et al., 2011*). Previous papers highlighted that SNAIL-1 subcellular localization and degradation is highly dependent on GSK-3β: GSK-3β is a kinase that mediates the phosphorylation of SNAIL-1 within the nucleus. SNAIL-1 is a transcriptional factor that is active in non-phosphorylated form, thus downregulating the expression of E-Cadherin. In the presence of phosphorylation due to GSK-3β, SNAIL-1 migrates to the outside of the nucleus and is degraded by the Ubiquitine-Proteasome system. In short, GSK-3β has the function of avoiding downregulation of E-Cadherin due to SNAIL-1 (*Zhou et al. 2004*). On the contrary, GSK-3β phosphorylation allows SNAIL-1 to accumulate into the nucleus, where it down-regulates E-cadherin gene.
In our experimental model, to assess the role of both GSK-3β and SNAIL-1, we performed time-dependence experiments (data not shown) where MeT-5A cells are incubated with TGFβ.In order to speed up the experiments, it was decided to perform the time dependency experiments, using directly the TGF-β solution and not the chrysotile suspension. A 6 h exposure to TGFβ resulted in GSK-3β phosphorylation and SNAIL-1 accumulation in the nucleus (Figure 4.9).

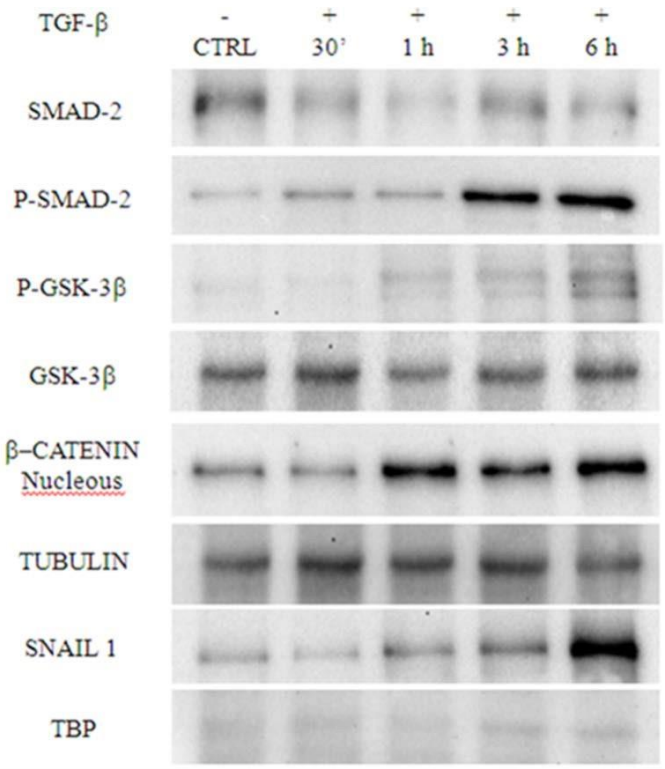


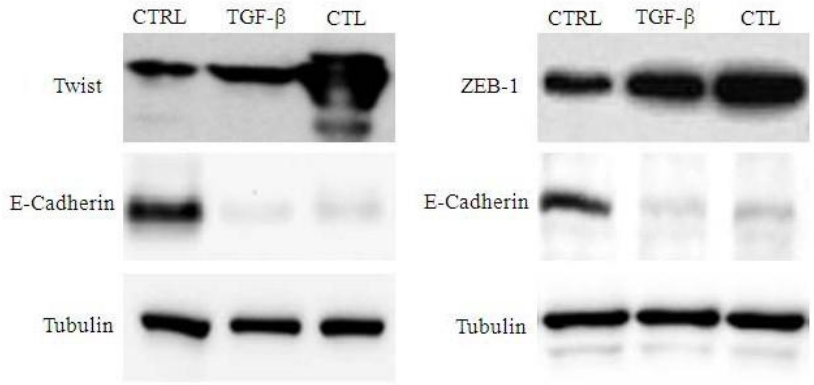
Figure 4.9:E-Cadherin gene down-regulation depends on the activation of the GSK-3β/Snail-1 pathway. MeT-5A cells were incubated in the absence or presence of10 ng/µl TGF-β for 0,5-1-3-6 h. The expression of phosphorylated GSK-3β (pGSK-3β) and the accumulation of SNAIL-1 in the nuclei of MeT-5A cells were examined by Western Blotting.

As can be seen from blot results and densitometric histograms, as for P-GSK-3β, we demonstrated an increase at 6 h incubation, but it starts at 1h. Additionally, an increase in P-SMAD signal at 3h and 6h incubation times overlaps with the previous P-GSK-3β data. It is to be noted, however, that a β-Catenin signal increase in the nucleus at the time of 1-3-6 h. Finally, according to the timings, displayed for P-GSK-3β and P-SMAD-2, there is also an increase in the SNAIL-1 signal at 6 hincubation.

Role of TWIST and ZEB-1 factors in EMT

The intracellular activation pathway of TGFβ may involve SMAD factors, which, in turn, mediate the activation of a series of signaling effectors associated with EMT. These effectors are grouped into families of factors such as SNAIL (including SNAIL-1 and SNAIL-2), the ZEB factor family (including ZEB-1 and ZEB-2) and the bHLH family (including E12, E47, TWIST and Ids) (*Peinado H. et al., 2007*). All these families have E-Cadherin gene as one of the final targets and SNAIL, ZEB or TWIST activation via TGFβ induces E-Cadherin downregulation. As shown above, SNAIL mediates E-Cadherin downregulation, then it is conceivable to hypothesize a role also for TWIST and ZEB-1 factors in EMT mediated by chrysotile fibers. Moreover TWIST and ZEB have been demonstrated to be markers of EMT in malignant mesothelioma (MM) (*Merikallio H. et al.,2013*).

In our experiments in MeT-5A cells incubated with TGFβ solution or chrysotile suspension for 72 hours, we can observe that both TWIST and anti-ZEB-1 showed an increased protein expression in comparison with untreated cells. At the same time, we can observe a downregulation of E- cadherin, thus confirming the EMT event (Figure 4.10).



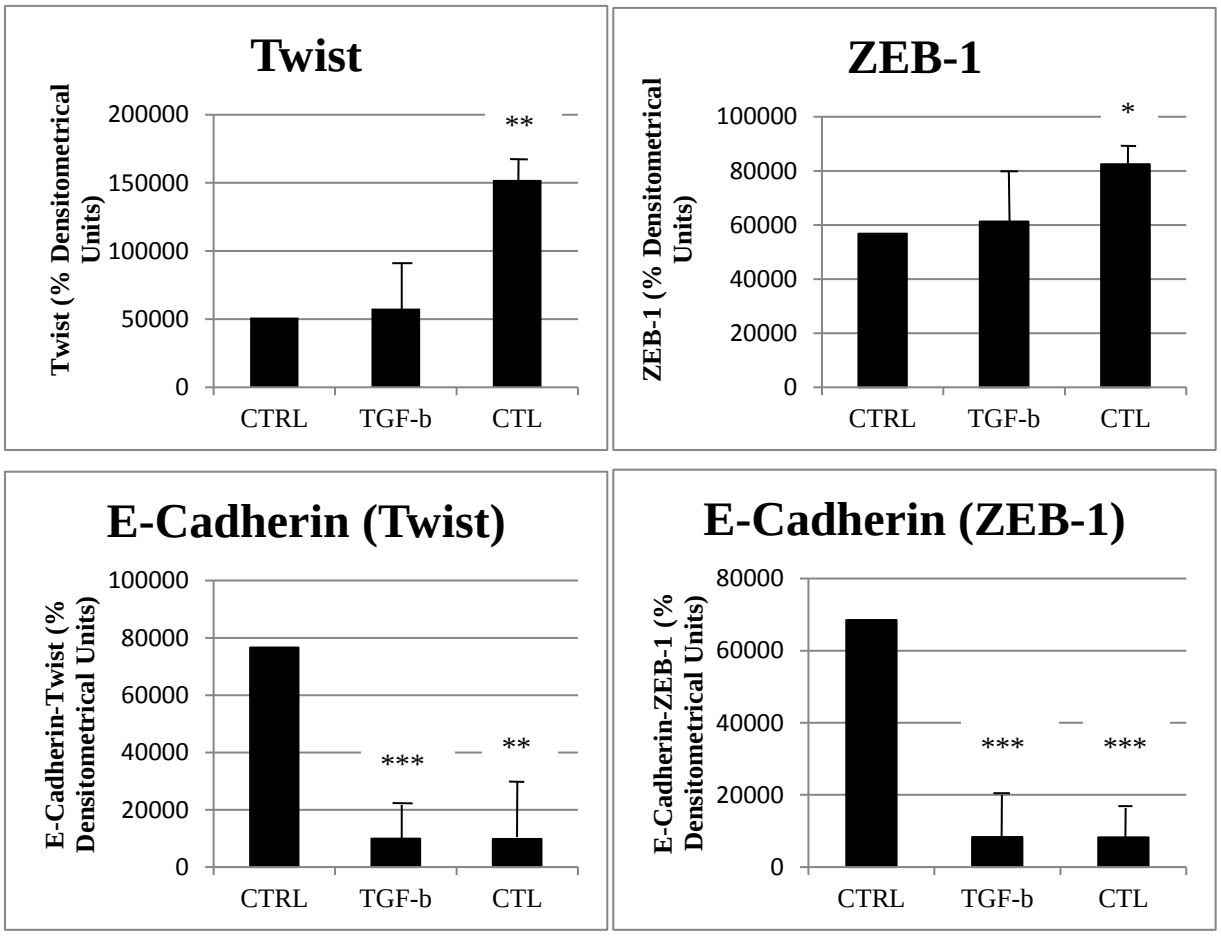


Figure 4.10: The images show western blot analysis performed on Twist and ZEB-1 factors. In the presence of 10 ng/µl TGFβ solution or 5 µg/cm^2 Chrysotile suspension (CTL), both Twist and ZEB-1 increases in MeT-5A, confirming also a repression of the expression of E-Cadherin. Significance *vs* respective CTRL:* $\rho<0,02$** $\rho<0,001$*** $\rho<0,0001$.

5. Discussion

In this thesis the topic is the study of molecular mechanisms, elicited by asbestos, which drive in mesothelial cells pathways that could be involved in the pathogenesis of asbestos-related diseases, such as fibrosis and malignant mesothelioma (Hodgson and Darnton, 2000). In all these pathological conditions, a common multistep event, called Epithelial-Mesenchymal Transition (EMT) has been demonstrated to play a fundamental role (*Kalluri and Weinberg, 2009*).

EMT is known to be a process that can be triggered by many different molecular mechanisms, and in many cellular models. TGFβ is one of the main EMT inducers and it has been already reported to mediate asbestos-induced fibrosis and inflammation *(Pociask et al., 2004*; *Sullivan et al., 2008*). However, despite these important evidences, the literature has not yet elucidated a clear mechanism connecting asbestos effects and EMT induction.

Few papers in literature focused on the ability of asbestos fibers to induce EMT. In 2012, Tamminen et al. carried out a study on A549 epithelioma cells by using crocidolite asbestos, and authors demonstrated crocidolite is able to induce EMT through a mechanism involving the MAPK/Erk signaling pathway, without a clear involvement of TGFβ. In another work, Kim et al. (2013) analyzed ROS-induced EMT in human malignant mesothelioma cells, suggesting that oxidative stress induced by H_2O_2 may play a critical role in mesothelioma carcinogenesis, via TGFβ, hypoxia inducible factor-1α and some stemness genes (Kim et al., 2013). Moreover, Batra et al. (2014) observed in a mouse model of lung fibrosis that, in response to TGFβ, pleural mesothelial cells lost their polarity and underwent phenotypic transition into myofibroblasts (Batra and Antony, 2014). Finally, Qi and co-workers (2013) observed chrysotile has limited transforming potential *in vitro* compared with crocidolite, as reflected by the number of tridimensional foci they observed in mesothelial cells. They also stated that the morphological and molecular alterations induced by both crocidolite and chrysotile asbestos are suggestive of EMT, and that chrysotile asbestos induces transient effects because of its different biopersistence compared to crocidolite (Qi et al., 2013).

In epithelial cells, E-Cadherin is known to mediate cell-cell tight junctions that are stabilized by β-Catenin (*Stappert and Kemler, 1994*): the loss or downregulation of E-Cadherin during EMT results in destabilization of the Cadherin/Catenin complex and disassembly of adherents junctions (*Pectasides et al., 2014*). In our cellular modelMeT-5A human mesothelial cells, chosen because are the main target of asbestos fibres, the expression levels of E-Cadherin significantly decreased after chrysotile asbestos exposure,and also β-Catenin protein levels resulted decreased, by confirming the loss of epithelial characteristics of MeT-5A cells.At the same time, mesenchymal proteins such as fibronectin, α-SMA and Vimentin were found to be

increased, suggesting the onset of cytoskeleton-related rearrangements typical of EMT (*Lamouille et al., 2014*). Furthermore, the increased deposition of fibronectin and the extrusion of MMP-2 in cells exposed to chrysotile asbestos suggest important changes in the surrounding microenvironment that make the extracellular matrix more suitable to be degraded and able to invade surrounding tissues (*Peinado et al., 2003*).

As previously reported, our research group demonstrated chrysotile asbestos induced TGFβ secretion in bronchial epithelial BEAS-2B cells (*Gulino et al., 2016*), thus promoting EMT event. Gulino et al. (2016) demonstrated the activation of TGFβ pathway induced by chrysotile, and this event drived EMT in pulmonary cells. In Met-5A cells, we demonstrated a strong production of TGFβ after asbestos incubation: there is a significant increase of TGFβsecretion after chrysotile exposure and, at the same time, cells developed a morphology suggestive of EMT. TGFβ involvement, and its correlation with EMT, was confirmed in our cellular model by using an TGFβ neutralizing antibody. The blocking antibody restored the expression levels of some epithelial and mesenchymal markers induced by chrysotile alone, thus suggesting that TGFβ can be considered the mediator of the reported EMT-related alterations.

To increase our knowledge about the molecular mechanisms involved in EMT chrysotile-mediated event, we referred to previous works in literature reporting TGFβ to be responsible for the up-regulation of the transcription factor SNAIL-1, that is in turn implicated in E-Cadherin gene downregulation (*Gulino et al., 2016*). To exert this function, SNAIL-1 persistence into the nucleus is required (*Zhou et al., 2004*). We observed a time-dependent accumulation of SNAIL-1 into the nucleus of MeT-5A cells incubated with TGFβ. This event may be result of the phosphorylation of GSK-3β, an ubiquitously expressed serine/threonine kinase that is a crucial in many cellular functions, contributing to the regulation of apoptosis, cell cycle, cell polarity and migration, gene expression, and many other functions, including the response to inflammatory stimuli (*Jope et al., 2007*).

We detected an increased phosphorylation of GSK-3β accompanied by the simultaneous accumulation of SNAIL-1 in the nucleus. With the present data we demonstrated for the first time that chrysotile asbestos induces EMT in MeT-5A cells with a molecular mechanism involving TGFβ and its intracellular effectors Akt/GSK-3β/SNAIL-1. However, the non-total recovery of the investigated epithelial and mesenchymal markers and of the intracellular mediators can be explained considering TGFβ as just one of the possible mediators of the asbestos-induced EMT event in these cells.

The obtained results confirm that asbestos is a very complex stimulus that can exert many

different cellular responses and involves a great amount of molecular pathways. Thus, it is likely that different multiple mechanisms may be activated as a consequence of asbestos exposure. Our data suggest that chrysotile asbestos is able to trigger EMT by involving TGFβ and its intracellular effectors Akt/GSK-3β/SNAIL-1, but also that this can be considered as just one of the possible molecular mechanisms supporting the morphological transformations typical of EMT involved in asbestos-related diseases.

As is well known, many signal molecules and signal transduction pathways are involved in EMT. *Kobayashi's et al.(2013)* reported that several pathways, including Wnt, TGF and ERK affect SNAIL expression during tumor progression. Consequently, Kobayashi's et al.(2013) suggest nuclear SNAIL expression plays a role in the production of more aggressive tumor cells in MM. Therefore, SNAIL could be a potential candidate for molecularly targeted therapy for MM, and the inhibition of SNAIL using small molecules or short hairpin RNA vectors could be a new treatment strategy for SNAIL-overexpressing MM.

In order to broaden our knowledge of other possible signaling pathways related to TGFβ, which could play a role in EMT and in correlation to MM development, we turned our attention to the transcription factors TWIST and ZEB-1, downstream factors as SNAILof the SMAD-mediated TGFβ signal transduction pathway, thus able to repress E-Cadherin (*Massague J et al., 2008*). Thus, both TWIST and ZEB,similar to SNAIL-1, are able to suppress E-Cadherin expression, thus contributing to the induction of EMT.

The two factors mentioned above, and the object of our research, have been shown to play a role in MM, as demonstrated by the research group of Merikallio H et al. (2012). In this work, they revealed that malignant mesothelioma has a higher TWIST and ZEB-1 increased expression. In our research we demonstrated an overexpression of both TWIST and ZEB-1 after chrysotile or TGFβ exposure, with in parallel the effective down-regulation of E-Cadherin. These results clearly demonstrated how TWIST and ZEB-1 are related to downregulation of E-Cadherin and induce EMT in MeT-5A cells, thus it is conceivable to hypothesize that this mechanism can be presumably involved in MM development, and EMT could be the event which could drive mesothelium tumor transformation. Taken as a whole, both asbestosis and MM are pathologies asbestos-related strongly associated to EMT event, and EMT markers could be considered useful in MM early diagnosis or in a therapeutic approach.

6.Conclusions and future perspectives

Results presented in this PhD thesis may be of great importance in clarifying the molecular mechanism in fibrotic and pro-carcinogenic effects of asbestos fibres. Exposure to asbestos and other toxic particulates has dramatically increased over the last century due to industrial and anthropogenic sources.
In conclusion, the results showed in this thesis demonstrate that the investigated markers can be useful to detect a first adverse response of cells to asbestos fibres in inducing pulmonary pathologies, in particular to elucidate the possible mechanisms which could drive the initiation or the progression of the asbestos correlated diseases, such as asbestosis or malignant mesothelioma.

Beside these important results, we focused on the fact that the number of factors able to affect asbestos pathological features ishuge, so, it is very important to study which of them are able to alter the pathogenic response of asbestos target cells.

Our future experiments will be focused in an attempt to elucidate if SNAIL, TWIST and ZEB-1 could drive mesothelial cells in the transformation into malignant mesothelioma, thus trying to confirm these markers as suitable genes predictive of the development of this severe pathology.

7. References

- Batra H, Antony VB. **The pleural mesothelium in development and disease.** Front Physiol 2014 Aug 1;5:284. doi: 10.3389/fphys.2014.00284. eCollection2014.
- Biscaldi G, Fonte R, Paita L, Vittadini G, Caprotti M. **High resolution computerized tomography in the diagnosis of asbestosis.** G Ital Med Lav Ergon. 1999 Oct-Dec;21(4):271-7. Italian. PMID:10771740
- Bourgeois P, Stoetzel C, Bolcato-Bellemin AL, Mattei MG, Perrin-Schmitt F (Dec 1996). **"The human H-twist gene is located at 7p21 and encodes a B-HLH protein that is 96% similar to its murine M-twist counterpart".**Mammalian Genome. 7 (12): 915–7. doi:10.1007/s003359900269. PMID8995765.
- Candura SM, Boeri R, Teragni C, Chen Y, Scala F. **Renal cell carcinoma abd malignant peritoneal mesotelioma after occupational exposure: case report**. Med Lav. 2016 May 26; 107(3):172-7.
- Casarsa C, Bassani N, Ambrogi F, Zabucchi G, Boracchi P, Biganzoli E, et al: **Epithelial-to-mesenchymal transition, cell polarity and stemness-associated features in malignant pleural mesothelioma.**Cancer Lett 2011; 302:136–143.
- Cichon MA1, Radisky DC.**ROS-induced epithelial to mesenchymal transition in mammary epithelial cells is mediated by NF-kB-dependent activation of Snail.**Oncotarget. 2014 May15;5(9):2827-38.
- Conway J, Al Zahrani KN, Pryce BR, Abou-Hamad J, Sabourin LA. **Transforming growth factor β–induced epithelial to mesenchymal transition requires the Ste20-like kinase SLK independently of its catalytic activity.** Oncotarget. 2017 Oct 19;8(58):98745-98756. doi: 10.18632/oncotarget.21928. eCollection2017.
- Cottin V, Brillet PY, Combarnous F, Duperron F, Nunes H, Cordier JF.**Syndrome of pleural and retrosternal "bridging" fibrosis and retroperitoneal fibrosis in patients with asbestos exposure.**Thorax. 2008 Feb;63(2):177-9. doi: 10.1136/thx.200X.081646.PMID:18234660
- Dumortier P, Thimpont J, de Maertelaer V, De Vuyst P.**Trends in asbestos body counts in bronchoalveolar lavage fluid over two decades.**Eur Respir J. 2003 Sep;22(3):519-24.PMID:14516145
- Fubini B,Hubbard A.**Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis.**Free Radic Biol Med2003; 34(12):1507-16.
- Gazzano E, Turci F, Foresti E, Putzu MG, Aldieri E, Silvagno F, Lesci IG, Tomatis M, Riganti C, Romano C, Fubini B, Roveri N, Ghigo D **Iron-loaded synthetic chrysotile:a**

new model solid for studying the role of iron in asbestos toxicity Chem Res Toxicol. 2007; 20(3):380-7.

- Ghio AJ, Kadiiska MB, Xiang QH, Mason RP. **In vivo evidence of free radical formation after asbestos instillation: an ESR spin trapping investigation**. Free Radic BiolMed. 1998 Jan 1;24(1):11-7.
- Gulino GR, Polimeni M, Prato M, Gazzano E, Kopecka J, Colombatto S, Ghigo D,Aldieri E. **Effects of Chrysotile Exposure in Human Bronchial Epithelial Cells: Insights into the Pathogenic Mechanisms of Asbestos-Related Diseases.**Environ Health Perspect. 2016 Jun;124(6):776-84. doi: 10.1289/ehp.1409627. Epub 2015 Dec 18.
- Hardy JA,Aust AE.**Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation andfibrosis.**
- Free Radic Biol Med. 2003 Jun15;34(12):1507-16.
- Huber MA, Beug H, Wirth T (Dec 2004). **"Epithelial-mesenchymal transition: NF-kappaB takes center stage"**. Cell Cycle. 3 (12): 1477–80. doi:10.4161/cc.3.12.1280. PMID 15539952.
- Iwai K, Adachi S, Takahashi M, Möller L, Udagawa T, Mizuno S, Sugawara I.**Early oxidative DNA damages and late development of lung cancer in diesel exhaust-exposed rats.**Environ Res. 2000 Nov;84(3):255-64.PMID:11097799
- Masui K1, Tanaka K, Akhavan D, Babic I, Gini B, Matsutani T, Iwanami A, Liu F, Villa GR, Gu Y, Campos C, Zhu S, Yang H, Yong WH, Cloughesy TF, Mellinghoff IK, Cavenee WK,Shaw RJ, Mischel PS. **mTOR complex 2controls glycolytic metabolism in glioblastoma through FoxO acetylation and upregulation of c-Myc.** Cell Metab. 2013 Nov 5;18(5):726-39. doi: 10.1016/j.cmet.2013.09.013. Epub 2013 Oct17.
- Jope RS, Yuskaitis CJ, Beurel E.**Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics.**Neurochem Res. 2007;32(4-5):577-95.
- Kalluri R, Weinberg RA (2009). **"The basics of epithelial-mesenchymaltransition"**. Journal of Clinical Investigation. **119** (6): 1420–1428. doi:10.1172/JCI39104. PMC 2689101 . PMID 19487818.
- Kamp DW, Graceffa P, Pryor WA, Weitzman SA. **The role of free radicals in asbestos-induced diseases**. Free Radic Biol Med, 1992; 12:293–315.
- Katoh Y, Katoh M (Sep 2008). **"Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA"**. Int J Mol Med. **22** (3): 271–5.

- Kim MC, Cui FJ, Kim Y.**Hydrogen peroxide promotes epithelial to mesenchymal transition and stemness in human malignant mesothelioma cells.**Asian Pac J Cancer Prev. 2013;14(6):3625-30.
- Kobayashi M, Huang CL, Sonobe M, Kikuchi R, Ishikawa M, Imamura N, Kitamura J, Iwakiri S, Itoi K, Yasumizu R, Date H. **Snail expression is associated with a poor prognosis in malignant pleural mesotheliomas.** Ann Thorac Surg. 2013 Apr;95(4):1181-8. doi: 10.1016/j.athoracsur.2013.01.012. Epub 2013 Mar1.
- Koskinen K, Pukkala E, Reijula K, Karjalainen A.**Incidence of cancer among the participants of the Finnish Asbestos Screening Campaign.**Scand J Work Environ Health. 2003Feb;29(1):64-70.PMID:12630438.
- Lamouille S,Xu J,Derynck R.**Molecular mechanisms of epithelial-mesenchymal transition.**Nat Rev Mol Cell Biol. 2014;15(3):178-96.
- Liu JY, Brody AR. 2001. **Increased TGF-beta1 in the lungs of asbestos-exposed rats and mice: reduced expression in TNF-alpha receptor knockout mice.** J Environ Pathol Toxicol Oncol20(2):97–108.
- Liu W, Huang YJ, Liu C, Yang YY, Liu H, Cui JG, Cheng Y, Gao F, Cai JM, Li BL (2014). **"Inhibition of TBK1 attenuates radiation-induced epithelial-mesenchymal transition of A549 human lung cancer cells via activation of GSK-3β and repression of ZEB1"**. Lab. Invest. **94** (4): 362–70. doi:10.1038/labinvest.2013.153.PMID24468793.
- Manning CB, Vallyathan V, Mossman BT. 2002. **Diseases caused by asbestos: mechanism of injury and disease development.** Int Immunopharmacol2(2–3):191–200.
- Heta Merikallio, Paavo Paakko, Kaisa Salmenkivi, Vuokko Kinnula, Teihu Harju, Ylermi Soini. **Expression of snail, twist, and Zeb1 in malignant mesothelioma.**APMIS©2012 APMIS DOI10.1111/j.1600-0463.2012.02931.x
- McDonald A, McDonald J, Pooley F. **Mineral fibre content of lung in mesothelial tumours in North America**. Ann Occup Hyg, 1982; 26:417–422.
- Miserocchi G1, Sancini G,Mantegazza F,Chiappino G.**Traslocation pathways for inhalated asbestos fibres**. Environ Health. 2008 Jan 24;7:4. doi:10.1186/1476-069X-7-4.
- Mohandas TK, Chen XN, Rowe LB, Birkenmeier EH, Fanning AS, Anderson JM, Korenberg JR (December 1995). **"Localization of the tight junction protein gene TJP1 to human chromosome 15q13, distal to the Prader-Willi/Angelman region, and to mouse chromosome 7".** Genomics. 30 (3): 594–7. doi:10.1006/geno.1995.1281. PMID8825647.
- Montserrat N1, Mozos A, Llobet D, Dolcet X,Pons C,de Herreros AG,Matias-Guiu X, Prat J. **Epithelial to mesenchymal transition in early stage endometrioidendometrial**

carcinoma. Hum Pathol. 2012 May;43(5):632-43. doi: 10.1016/j.humpath.2011.06.021. Epub 2011 Sep 21.

- Murthy S, Larson-Casey JL, Ryan AJ, He C, Kobzik L, Carter AB. 2015. **Alternative activation of macrophages and pulmonary fibrosis are modulated by scavenger receptor, macrophage receptor with collagenous structure.** FASEB J29(8):3527–3536.
- Nagamoto T1, Eguchi G, Beebe DC.**Alpha-smooth muscle actin expression in cultured lens epithelial cells.** Invest Ophthalmol Vis Sci. 2000Apr;41(5):1122-9.
- Niccoli-Asabella A, Notaristefano A, Rubini D, Altini C, Ferrari C, Merenda N, Fanelli M, Rubini G. **18F-FDG PET/CT in suspected recurrences of epithelial malignant pleural mesothelioma in asbestos-fibers-exposed patients (comparison to standard diagnostic follow-up).**Clin Imaging. 2013 Nov-Dec;37(6):1098-103. doi: 10.1016/j.clinimag.2013.06.009. Epub 2013 Aug8.PMID:23932389
- Nouri M, Ratther E, Stylianou N, Nelson CC, Hollier BG, Williams ED (2014).**"Androgen-targeted therapy-induced epithelial mesenchymal plasticity and neuroendocrine transdifferentiation in prostate cancer: an opportunity for intervention".**Front Oncol. **4**: 370. doi:10.3389/fonc.2014.00370.PMC 4274903. PMID25566507.
- Pankov R, Yamada KM (Oct 2002). **"Fibronectin at a glance".** Journal of CellScience. 115 (Pt 20): 3861–3. doi:10.1242/jcs.00059. PMID 12244123.
- Paznekas WA, Okajima K, Schertzer M, Wood S, Jabs EW (November 1999). **"Genomic organization, expression, and chromosome location of the human SNAIL gene (SNAI1) and a related processed pseudogene (SNAI1P)".**Genomics. 62 (1):42–9. doi:10.1006/geno.1999.6010. PMID 10585766.
- Pectasides E,Rampias T,Sasaki C,Perisanidis C,Kouloulias V,Burtness B, Zaramboukas T,Rimm D, Fountzilas G,Psyrri A. **Markers of epithelial to mesenchymal transition in association with survival in head and neck squamous cell carcinoma (HNSCC)**. PLoS One. 2014;9(4):e94273.
- Peinado H,Quintanilla M,Cano A.**Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions.** J Biol Chem. 2003;278(23):21113-23.
- Peinado H, Olmeda D, Cano A (2007). **"Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?"**. Nature Reviews Cancer. **7** (6): 415–428. doi:10.1038/nrc2131.PMID17508028.

- Phua, YL; Martel, N; Pennisi, DJ; Little, MH; Wilkinson, L (April 2013). **"Distinct sites of renal fibrosis in Crim1 mutant mice arise from multiple cellular origins.".** The Journal of pathology. **229** (5): 685–96. doi:10.1002/path.4155. PMID23224993.
- Pociask DA,Sime PJ, Brody AR.**Asbestos-derived reactive oxygen species activate TGF-beta1**. Lab Invest 2004;84(8):1013-23.
- Qi F, Okimoto G, Jube S, Napolitano A, Pass HI, Laczko R, Demay RM, Khan G, Tiirikainen M, Rinaudo C, Croce A, Yang H, Gaudino G, Carbone M. **Continuous exposure to chrysotile asbestos can cause transformation of human mesothelial cells via HMGB1 and TNF-α signaling.**Am J Pathol. **2013** Nov;183(5):1654-66. doi: 10.1016/j.ajpath.**2013**.07.029.
- Rahman I,Biswas SK,Kode A.**Oxidant and antioxidant balance in the airways and airway diseases**. Eur J Pharmacol. 2006;533(1-3):222-39.
- Rascu A, Naghi E, O Telea MR, NiTu FM, Arghir OC. **Distinction between mesotelioma and lung adenocarcinoma based on immunoistochemistry in a patient with asbestos bodies in broncoalveolar fluid-case report.** Rom J Morphol Embryol. 2016;57(3): 1171-1174.
- Rhyu DY,Yang Y,Ha H,Lee GT,Song JS, Uh ST,Lee HB.**Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells**. J Am Soc Nephrol.2005;16(3):667-75.
- Schramm A, Opitz I, Thies S, Seifert B, Moch H, Weder W, et al: **Prognostic significance of epithelial-mesenchymal transition in malignant pleural mesothelioma.** Eur J Cardiothorac Surg 2010; 37:566–572.
- Schuberth PC, Hagedorn C, Jensen SM, Gulati P, van den Broek M, Mischo A, Soltermann A, Jüngel A, Marroquin Belaunzaran O, Stahel R, Renner C, Petrausch U**. Treatment of malignant pleural mesothelioma by fibroblast activation protein-specific re-directed T cells.**J Transl Med. 2013 Aug 12;11:187. doi: 10.1186/1479-5876-11-187.PMID:23937772
- Stappert J, Kemler R. **A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated.**Cell Adhes Commun 1994;2(4):319-27.
- Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA (September 1986). **"Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia".**The Journal of Cell Biology. 103 (3): 755–66. doi:10.1083/jcb.103.3.755. PMC 2114282 . PMID3528172.

- Sullivan DE,Ferris M,Pociask D, Brody AR. **The latent form of TGFbeta(1) is induced by TNFalpha through an ERK specific pathway and is activated by asbestos-derived reactive oxygen species in vitro and in vivo.**J Immunotoxicol 2008;5(2):145-9.
- Tamminen JA, Myllärniemi M, Hyytiäinen M,Keski-Oja J,Koli K. **Asbestos exposure induces alveolar epithelial cell plasticity through MAPK/Erk signaling.**J Cell Biochem 2012;113(7):2234-47.
- Thiery JP, Acloque H, Huang YJ, Nieto MA (2009). **"Epithelial-Mesenchymal Transitions in Development and Disease"**. Cell. **139** (5): 871–890. doi:10.1016/j.cell.2009.11.007.
- Valavanidis A,Vlachogianni T,Fiotakis K, Loridas S.**Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms.**Int J Environ Res Public Health. 2013;10(9):3886-907.
- Villarejo A, Cortés-Cabrera A, Molina-Ortíz P, Portillo F, Cano A (January 2014). **"Differential role of Snail1 and Snail2 zinc fingers in E-cadherin repression and epithelial to mesenchymal transition".** The Journal of Biological Chemistry. 289 (2): 930–41. doi:10.1074/jbc.M113.528026. PMC 3887216 . PMID24297167.
- Vlahopoulos SA, Logotheti S, Mikas D, Giarika A, Gorgoulis V, Zoumpourlis V (Apr 2008). **"The role of ATF-2 in oncogenesis".** BioEssays. **30** (4): 314–27. doi:10.1002/bies.20734. PMID18348191.
- Williams CM, Engler AJ, Slone RD, Galante LL, Schwarzbauer JE (May 2008). **"Fibronectin expression modulates mammary epithelial cell proliferation during acinar differentiation".** Cancer Research. 68 (9): 3185–92. doi:10.1158/0008-5472.CAN-07-2673. PMC 2748963 . PMID18451144.
- Williams TM, Montoya G, Wu Y, Eddy RL, Byers MG, Shows TB (September 1992). **"The TCF8 gene encoding a zinc finger protein (Nil-2-a) resides on human chromosome 10p11.2".** Genomics. 14 (1): 194–6. doi:10.1016/S0888-7543(05)80307-6. PMID1427828.
- Wong SHM, Fang CM, Chuah LH, Leong CO, Ngai SC. **E-Cadherin: its dysregulation in carcinogenesis and clinical implications.** Crit Rev Oncol Hematol. 2018 Jan; 121:11-22. doi: 10.1016/j. critrevonc.2017.11.101. Epub 2017 Nov20.
- Xie X, Zheng X, Wang J, Chen L. **Clinical significance of Twist, E-Cadherin, and N-Cadherin protein expression in endometrioid adenocarcinoma.** J Cancer Res Ther. 2017;13(5):817-822. doi:10.4103/jcrtJCRT_405_17.

- Yang J, Weinberg RA (2008). **"Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis"**. Dev Cell. **14** (6):818–829. doi:10.1016/j.devcel.2008.05.009.PMID 18539112.
- Zhou BP,Deng J, Xia W,Xu J, Li YM,Gunduz M,Hung MC. **Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition.**Nat Cell Biol 2004;6(10):931-40.
- WHO, 1986
- NTP,2005

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