



Article

TGF- β Inhibition Through Combinatory Strategies Suppresses Proliferation and Invasiveness in Malignant Pleural Mesothelioma

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Abstract

Malignant pleural mesothelioma (MPM) is a rare and aggressive tumor associated with asbestos exposure, which represents a current problem. MPM is characterized by a poor prognosis and an unsatisfactory therapeutic approach. Therefore, improving MPM prognosis is the real challenge for research today. Regarding preclinical data, Transforming Growth Factor- β (TGF- β) plays a crucial role in cancer, and its alteration has been associated with tumor progression and invasiveness, in particular through the Epithelial to Mesenchymal Transition (EMT) event. We investigated the role of TGF- β inhibition in proliferation, cell cycle, migration, and invasiveness in human MPM cells. Data obtained clearly highlighted how TGF- β inhibition, through the silencing or treatment of MPM cells with antibody anti-TGF- β (Fresolimumab), significantly reduces cell proliferation (MTT, PCNA) and prevents metastasis, reducing EMT and decreasing the invasiveness and migration of MPM cells. Finally, we also evaluated TGF- β inhibitory effects in 3D MPM cellular models (spheroids), highlighting a significant slowdown in the growth rate of spheroids treated with anti-TGF- β antibody or Fresolimumab, confirming the results previously obtained. Taken as a whole, targeting TGF- β will represent a starting point for future improvements in MPM management. This is particularly important as we foresee a growing increase in MPM in the coming years.

Keywords: malignant pleural mesothelioma; Transforming Growth Factor- β (TGF- β); epithelial to mesenchymal transition; tumor proliferation; invasiveness

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1. Introduction

Malignant pleural mesothelioma (MPM) is a rare but very aggressive cancer associated with asbestos exposure of either professional or environmental origin [1]. To date, MPM still represents a current problem, and, in the last 25 years, there has been no significant increase in the median survival of MPM patients [2]. Indeed, MPM is a cancer

usually diagnosed at an advanced stage, with limited treatment options and a consequently worse prognosis. The current diagnosis of MPM is based on imaging modalities and the evaluation of diagnostic, such as mesothelin and BRCA-1-associated protein (BAP1) [3–5], and prognostic biomarkers. A current problem is the lack of an early diagnosis because MPM patients typically present one or more symptoms that are very unspecific [4]: this leads to diagnosis or surgical intervention occurring too late in advanced MPM. Until now, MPM has been characterized by an unfavorable prognosis and an unsatisfactory therapeutic approach, with a poor median survival of patients of less than 12 months if untreated [6,7]. Furthermore, increasing attention has been paid in recent years to the study of MPM, especially considering the growing incidence of this tumor in the recent and coming years [7].

Therapeutic approaches in the treatment of MPM consist of multiple modalities: surgery, chemotherapy, immunotherapy and radiotherapy [8]. Despite the effectiveness of the chemotherapeutic agents used (cisplatin and pemetrexed, with or without bevacizumab), these treatments cause chemoresistance, limiting their efficacy in counteracting MPM [9]. Recently, immunotherapy (immune checkpoint inhibitors, nivolumab, anti-programmed cell death 1) has been approved for the first-line treatment of unresectable MPM [10]. However, these approaches are still limited. To date, deep research into the molecular mechanisms involved in MPM pathogenesis has opened new scenarios with potential to improve the therapeutic target in MPM management.

The MPM pathogenesis differs from that of many other tumors since it is characterized by a very long latency phase [11], during which inhaled asbestos fibers induce a strongly inflammatory environment that can lead to both cell death and/or transformation [12]. The main hypothesis is that mesothelial cells, subjected to this unfavorable environment, progressively acquire the ability to grow under stressing conditions and become resistant to multiple stresses, such as oxidative stress and/or chemotherapeutics [13], thus developing and maintaining a favorable tumor microenvironment (TME).

In cancer cells, several cytokines and growth factors have been demonstrated to be involved in the development and progression of cancer. Among these, the Transforming Growth Factor- β (TGF- β) appears to be fundamental for tumorigenesis and cancer progression/invasiveness, although it depends on the stage of tumor itself [14]: in particular, concerning preclinical research, TGF- β has been shown to play a crucial role at the pulmonary level, both in physiological conditions, such as lung organogenesis, and in pathological ones, in particular oncological pathologies, such as lung cancer [15].

It has recently been observed that, in carcinogenesis process, frequent alterations at the TGF- β level are associated with the progression and invasiveness of cancer [14,15], especially through the induction and deregulation of the Epithelial to Mesenchymal Transition (EMT), a reversible event deeply involved in the onset, progression and metastasis of tumors [16], and of which TGF- β is the main promoter. In tumorigenesis, TGF- β secretion contributes to promoting cancer development and supporting the creation of a malignant TME, thus driving the creation of new vessels (angiogenesis), immune escape and stromal interactions [14,17]. In the advanced stages of malignancy, tumor invasiveness/metastasis has been shown to be strongly driven by TGF- β [18], particularly via the EMT event.

Growing scientific evidence suggests the crucial importance of TGF- β in the progression and invasiveness of MPM: most human mesothelioma cell lines tested secrete significant amounts of TGF- β and mesothelioma tissue samples showed strong immunoreactivity to TGF- β in the majority of samples tested [17]. Moreover, the blocking of TGF- β , using a small molecular inhibitor or a soluble receptor, has shown the significant inhibition of MPM growth [19]. Recently, a phase II study demonstrated the efficacy of Fresolimumab, a new anti-TGF- β antibody, against a small number of patients with advanced

mesothelioma when pre-treated with this drug, improving the average overall survival by 15 months [20], and, in a small cohort of MPM patients, TGF- β levels in their pleural effusions were significantly higher than levels present in lung cancer [21,22]. Finally, EMT signature is associated with a worst prognostic value in MPM [23] and, in MPM different histotypes, it has been demonstrated there is variable expression of cellular mesenchymal and epithelial markers [24], depending on the severity of prognosis.

However, it should not be forgotten that TGF- β performs an enormous number of functions. Nevertheless, TGF- β inhibition has been shown to block tumorigenesis *in vivo*, and inhibitors of TGF- β or the TGF- β receptor have been demonstrated to cause no major side effects, thus providing a good compromise between maximum therapeutic effects and minimum side effects [15]. Moreover, the TGF- β signature can be considered a predictive marker of worst prognosis of MPM, and TGF- β inhibition has been shown to be effective in reducing the growth of tumors, while also preventing metastasis [16].

Finally, concerning the therapeutic approach to MPM, some inhibitors of TGF- β and EMT (in turn mediated by TGF- β) could be used in combination with chemo- and immunotherapy, thus trying to improve current pharmacological therapy. Although the involvement of TGF- β in tumor progression and the potential benefit of its inhibition is a highly explored topic in cancer research, combination therapies will likely remain the focus, pairing TGF- β inhibitors with targeted therapies to maximize their effects on tumor progression and immune modulation. In our work, we demonstrated the importance of synergizing the MPM approach, targeting both TGF- β and its pathway via a reverting EMT event. Thus, we inhibited not only cancer proliferation but also the metastasis driven by this crucial cytokine.

2. Results

2.1. TGF- β Silencing and/or Inhibition Decrease MPM Cell Proliferation

Based on our previously published data [24], which demonstrated the role of TGF- β in driving EMT in human mesothelial and MPM cells, we explored the effect of TGF- β inhibition on MPM cell proliferation. The results obtained clearly showed increased cellular proliferation in MSTO-211H cells incubated with TGF- β (Figure 1A,B), while, at the same time, TGF- β siRNA transfection significantly inhibited MSTO-211H cellular proliferation (Figure 1A–C) towards untreated cells by significantly inhibiting PCNA (Proliferating Cell Nuclear Antigen), an antigen expressed in the nucleus during the DNA synthesis phase whose expression is directly proportional to cell proliferation, gene transcription (Figure 1B), and cell viability (Figure 1C). In Figure 1A, it is also evident how TGF- β treatment induced morphological changes in MPM cells typical for EMT-related events. Moreover, our results clearly show that MPM cellular proliferation has been demonstrated to be significantly inhibited after both TGF- β silencing and anti-TGF- β antibody cellular incubation (Figure 1C).

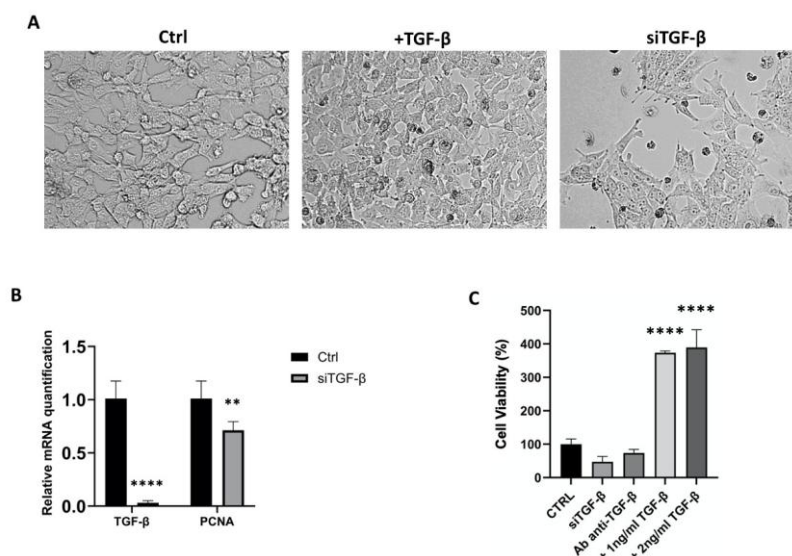


Figure 1. Effect of TGF- β inhibition on MPM cell proliferation. Evaluation of proliferation after 48 h of incubation with TGF- β (1–2 ng/mL), anti-TGF- β antibody (Ab anti-TGF- β) and/or TGF- β silencing (siTGF- β): cells were analyzed morphologically under a confocal microscope (**panel A**), and proliferation was measured by evaluating PCNA mRNA (**panel B**) and by MTT assay (**panel C**). All measurements were performed in triplicate. (**B**) Statistical significance is indicated as **** $p < 0.0001$ or ** $p < 0.01$ for treated cells compared to their respective untreated controls (Ctrl). (**C**) Statistical significance is indicated as **** $p < 0.0001$ for treated cells compared to their respective untreated controls (Ctrl).

Then, to confirm the results obtained on MSTO-211H cell line, we also performed experiments in MPM patient-derived cells. In particular, TGF- β silencing or anti-TGF- β antibody cells treatment were set up on three MPM histotypes: epithelioid (MM404), biphasic (MM421), and sarcomatous (MM432) histological types. TGF- β inhibition across all MPM cell lines following transfection with TGF- β siRNA (Figure 2A) also significantly confirms the efficacy of silencing in MPM patient-derived cells. So, our data clearly show the significant inhibition of cell proliferation (PCNA detection) (Figure 2B) and cell viability (Figure 2C) in all MPM cellular histotypes, although a little more in the biphasic one.

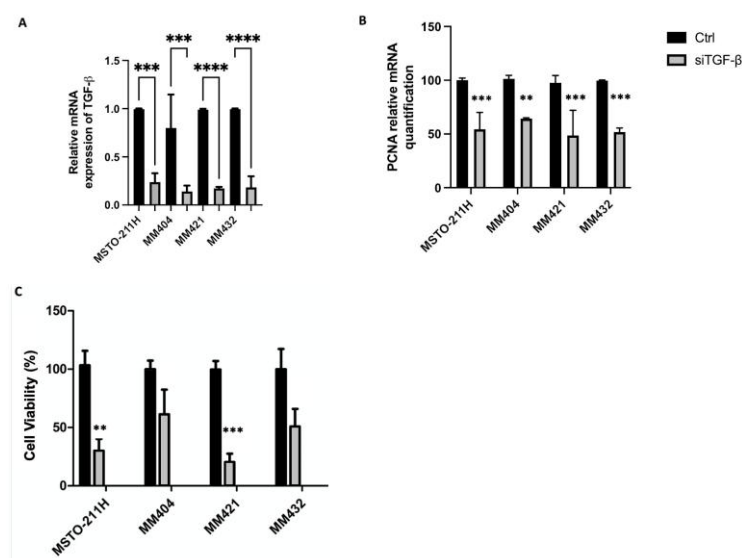


Figure 2. Efficacy of TGF- β silencing and its effect on cell viability and proliferation. (A) Percentage of TGF- β inhibition across all MPM cell lines (MSTO-211H, MM404, MM421, MM432) following

transfection with TGF- β siRNA (60 nM). Data are presented as means $\pm$ SD ($n = 3$). **** $p < 0.0001$, *** $p < 0.001$: siTGF- β vs. respective Ctrl. (B) Evaluation of the cell proliferation marker PCNA by real-time PCR 48 h after transfection. Data are presented as means $\pm$ SD ($n = 3$). *** $p < 0.001$, ** $p < 0.01$: siTGF- β vs. respective Ctrl. (C) Evaluation of cell viability via MTT assay 48 h after transfection with TGF- β siRNA (60 nM). Measurements were performed in triplicate. Data are expressed as percentage of viable cells relative to control and presented as means $\pm$ SD ($n = 3$). *** $p < 0.001$, ** $p < 0.01$: siTGF- β vs. respective Ctrl.

2.2. TGF- β Silencing and/or Inhibition Strongly Impair Migration Rate and Cell Cycle of MPM Cells

To better check the crucial role of TGF- β in driving MPM metastasis, we studied a possible effect of TGF- β silencing on cell migratory capacity and cell cycle perturbation. MSTO-211H cells, used as a representative MPM cell line, showed a significant reduction in the migratory capacity after TGF- β silencing, as shown in Figure 3A, and an impaired cell cycle (Figure 3B): TGF- β reduction led to an increased number of MPM cells arrested in the S phase, thus confirming a perturbation in MPM cell cycle by blocking TGF- β .

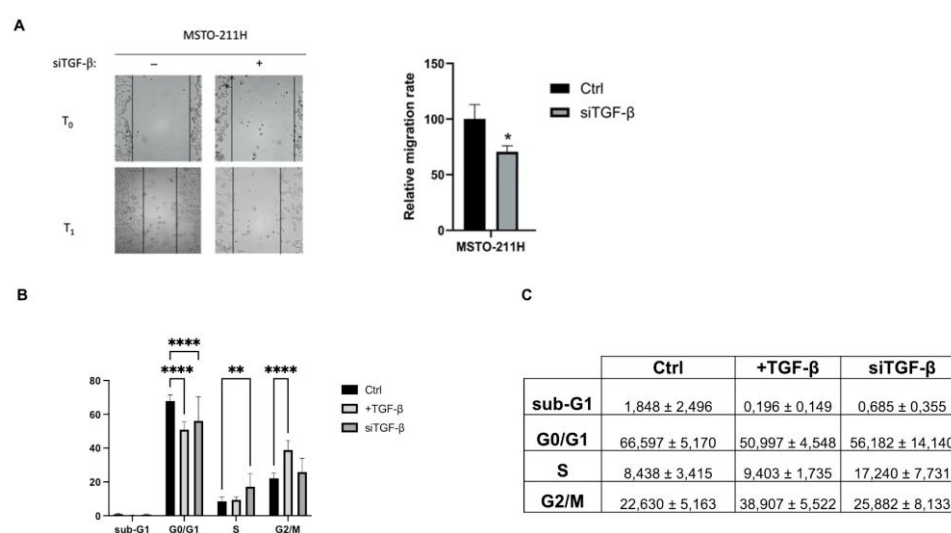


Figure 3. Effect of TGF- β silencing on MPM cell migration and cell cycle. (A) Scratch assay evaluated on MSTO-211H cells after 48 h without (-) or with 60 nM TGF- β siRNA. Cells were analyzed by microscopy at the time of scratching (T0) and after 24 h (T1). Relative migration was calculated by setting the percentage migration of control cells at T1 equal to 100% and comparing the percentage migration of cells after transfection with siTGF- β to this value. Data are presented as means $\pm$ SD. **** $p < 0.0001$: cells transfected with siTGF- β vs. Ctrl cells. (B) Cell cycle analysis using flow cytometry after staining with propidium iodide (PI). (C) Distribution of cells in sub-G1, G1/G0, S, and G2/M phases of the cell cycle under different experimental conditions. Data are presented as means $\pm$ SD ($n = 3$). **** $p < 0.0001$, ** $p < 0.01$ and * $p < 0.05$: treated cells vs. respective untreated cells (Ctrl).

2.3. TGF- β Silencing and/or Inhibition with Fresolimumab Reverts EMT in MPM Cells.

We investigated the effects of TGF- β silencing and/or inhibition using Fresolimumab (TGF- β -neutralizing antibody drug) [25] on EMT-related mechanisms driving metastasis in MPM. In particular, changes in E-cadherin expression support the observed impact on cell migration. As shown in Figure 4, the most effective treatments were siRNA transfection and the combined approach, both of which led to the marked upregulation of E-cadherin, indicating a partial reversal of EMT [Figure 4A]. Furthermore, immunofluorescence analysis confirmed these findings by demonstrating the concomitant downregulation of Fibronectin in MPM cell lines following TGF- β inhibition [Figure 4B], although it occurred

differently depending on the histotype. This further supports the notion of EMT reversion and suggests a shift toward a less aggressive cellular phenotype.

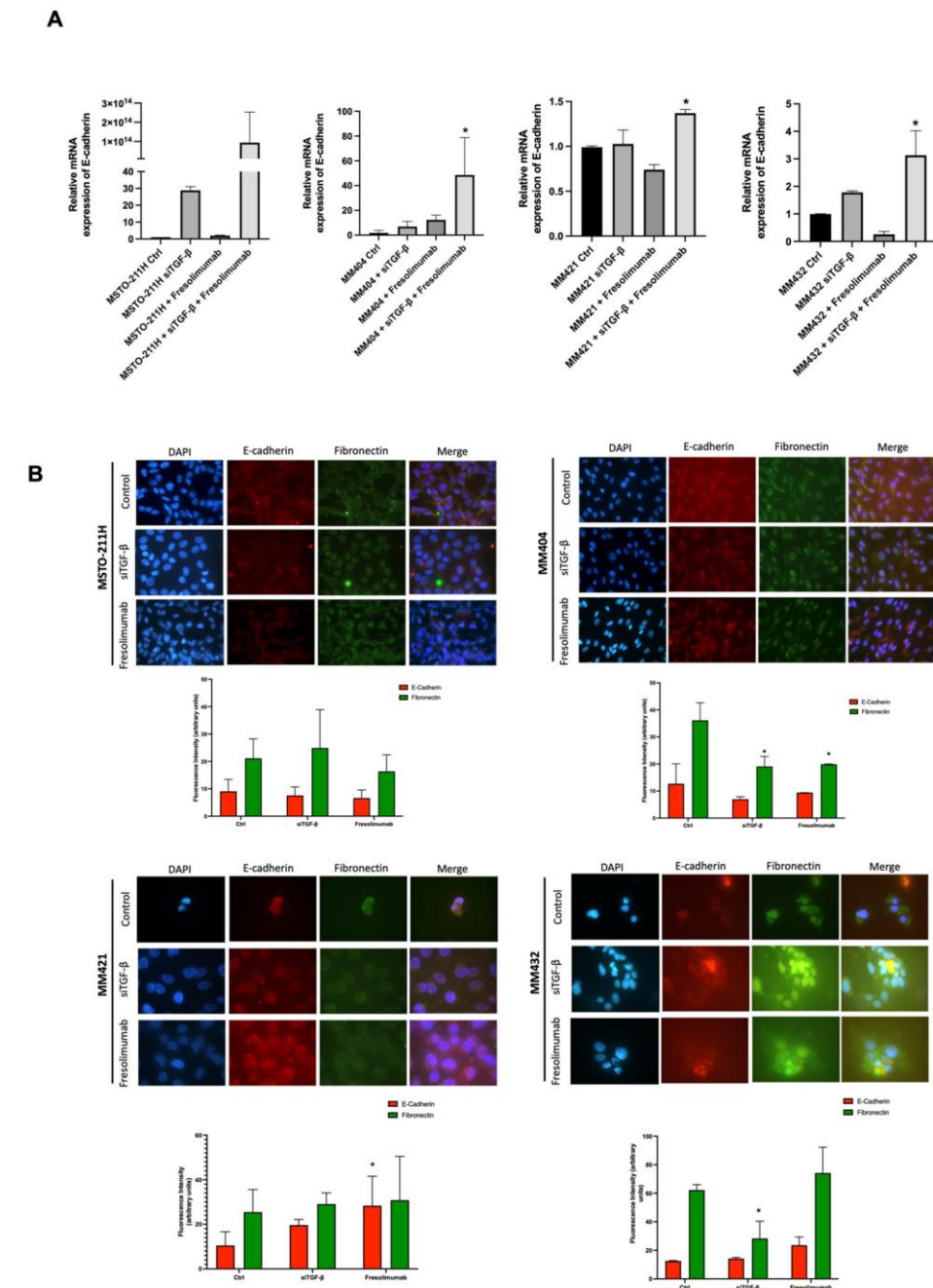


Figure 4. Effect of TGF- β inhibition on EMT in MPM cells. (A) E-cadherin expression, a key epithelial marker in EMT, was analyzed by real-time PCR in MPM cells treated with TGF- β siRNA (60 nM), Fresolimumab, or their combination. Measurements were performed in triplicate. $^{***} p < 0.001$ and $p < 0.01$ indicate treated cells compared to their respective untreated cells (Ctrl). (B) Representative confocal immunofluorescence images showing the localization and expression of E-cadherin and Fibronectin in treated and untreated cells. Cells were fixed, permeabilized, and stained with specific primary antibodies and then by fluorophore-conjugated secondary antibodies. E-cadherin is visualized in red (TRITC), and Fibronectin is shown in green (CFTM488A). Cell nuclei were counterstained with DAPI (blue). Images were acquired using a Leica DM IRB camera. The accompanying histograms represent the fluorescence intensity of the respective markers. Data are presented as mean $\pm$ SD ($n = 3$): $^{**} p < 0.01$ and $^{*} p < 0.05$ indicate treated cells compared to their respective untreated cells (Ctrl).

2.4. TGF- β Inhibition Strongly Slows MPM Spheroid Growth

To better demonstrate our data in a 3D tumor model, we evaluated the effect of TGF- β block in MPM (MSTO-211-H, M404, M421, M432)-derived spheroids, obtained after three days by seeding cells in an ultra-low-adhesion plate, as described in the Materials and Methods section.

We then evaluated the correlation between TGF- β inhibition, by incubation with anti-TGF- β antibody, and the proliferation rate of spheroids. As shown in Figure 5A,B, we observed a significant slowing of cell growth rate after 48 h of incubation with the anti-TGF- β antibody compared to untreated spheroids, with a significant reduced proliferation rate in all treated cells (Figure 5A,B).

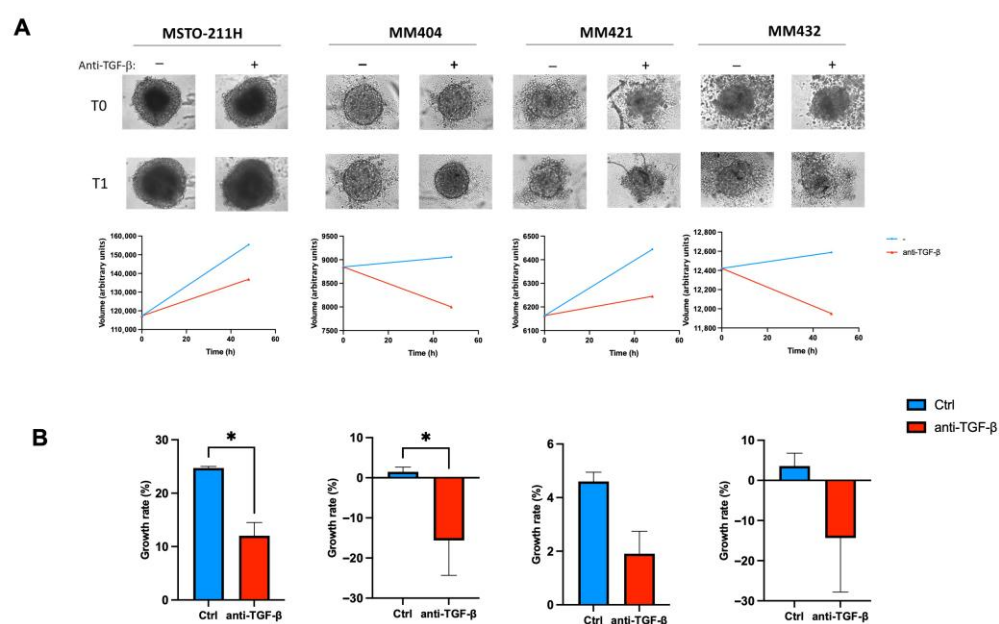


Figure 5. Effect of TGF- β inhibition on MPM spheroids. Evaluation of the volume of MPM spheroids after 48 h untreated (-) or treated with 2 ng/mL of anti-TGF- β . The spheroids were analyzed by microscopy at time T0 and 48 h after incubation with anti-TGF- β (T1). The volume was measured in arbitrary units over 48 h (**panel A**) and the growth rate was measured by setting the volume of untreated (-) spheroids at T1 equal to 100% towards spheroids incubated with anti-TGF- β (**panel B**). The experiments were performed in triplicate with statistical significance set at * $p < 0.05$.

2.5. Fresolimumab Inhibits MPM Spheroid Growth Synergizing with siRNA Treatment.

As our previous data showed that blocking TGF- β reduced growth, we explored the efficacy of the anti-TGF- β drug Fresolimumab and/or TGF- β silencing in our MPM spheroids: our results confirmed that Fresolimumab, like TGF- β silencing, is an effective inhibitor in significantly inhibiting spheroid growth in all MPM spheroids. Furthermore, we also showed a synergistic effect between Fresolimumab and siRNA treatment [Figure 6].

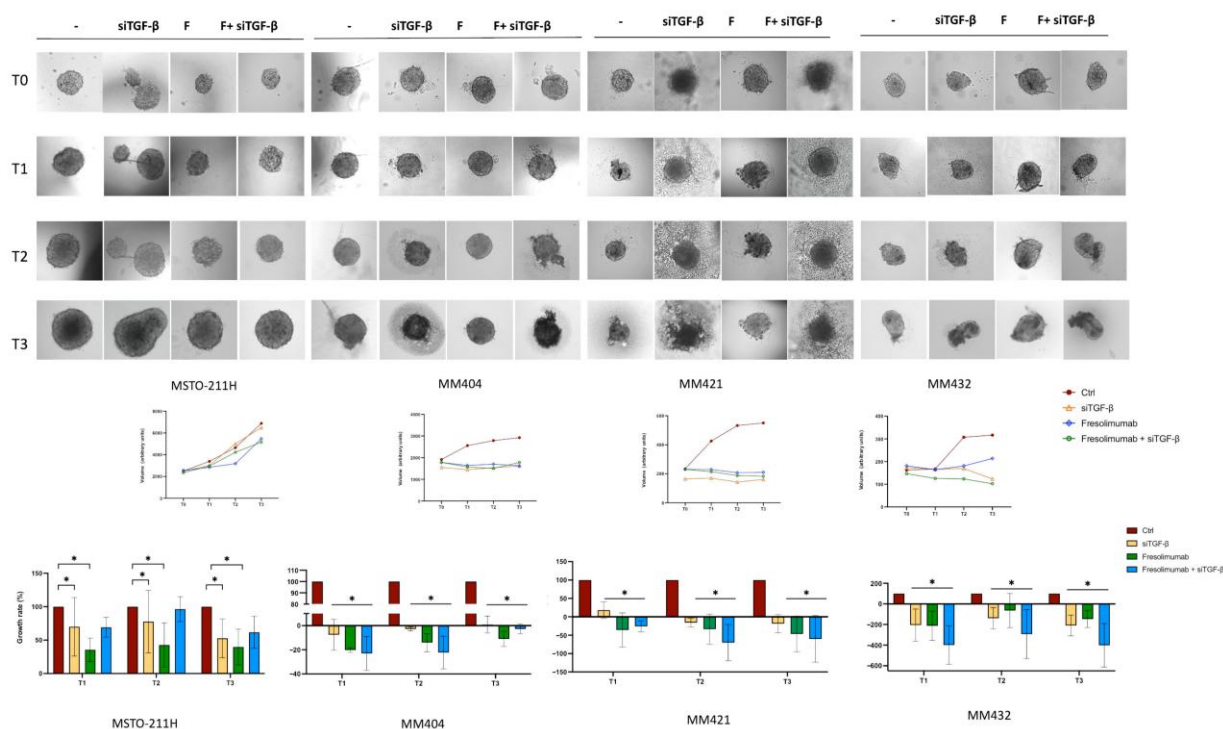


Figure 6. Effect of Fresolimumab and siRNA treatments on MPM spheroid growth. 3D spheroid models were generated by seeding MPM cells into Ultra-Low Attachment plates and treated with siRNA against TGF- β (siTGF- β), Fresolimumab, or their combination after 3 days. Spheroid volume was monitored at 24 h (T1), 48 h (T2), and 72 h (T3) and measured in arbitrary units (**panel A**). Growth rate by setting the volume of untreated (-) spheroids at T1 equal to 100% towards treated spheroids (**panel B**). Experiments were performed in triplicate with statistical significance set at and * $p < 0.05$.

3. Discussion

The current problem of asbestos-related diseases, such as MPM, is the lack of sensitive prognostic markers or pharmacological targets useful for improving the therapeutic approach, particularly given the aggressiveness, resistance to chemotherapy treatment, and poor prognosis of this type of cancer. To date, a number of published works in the literature have studied the potential of TGF- β as a pharmacological target in tumor diseases [14,15,18,23,26,27] but, despite the existence of preclinical studies and translational research demonstrating that TGF- β blockade is a potentially effective therapeutic strategy [15,27,28], the translation from the bench to the bedside has been slow and to date still not fully successful. Recently, some studies have also focused on the possible use of TGF- β as a diagnostic and prognostic marker: Paul Stockhammer and Till Ploenes [19] claimed that MPM patients had significantly higher pleural effusion TGF- β levels compared to lung or breast cancer patients, making TGF- β a possible marker for differential diagnosis. However, although none of the TGF- β inhibitors tested in clinical trials have received regulatory approval for cancer therapy to date, several agents (antibody-based and small-molecule inhibitors) are currently under investigation in ongoing clinical trials across various cancer types, including mesothelioma. However, these efforts have encountered challenges related to safety (e.g., immune suppression) and have yielded mixed results regarding clinical efficacy [23].

The aim of this work has been addressed to clarify how TGF- β is involved in the carcinogenesis and metastasis of MPM: in our study, we are targeting TGF- β to explore

how inhibiting this cytokine and its pathway could be effective in reducing MPM growth and invasiveness. We aim to achieve this last objective via inhibition and reverse EMT.

Starting at this point, we evaluated whether, when blocking TGF- β via silencing TGF- β or through incubation with an anti-TGF- β antibody, there was a reduction in MPM cell proliferation. Firstly, in our MPM cellular models, we demonstrated TGF- β is effective in promoting proliferation when incubated in MPM cells and, at the same time, its silencing or inhibition strongly and significantly inhibits MPM cells growth, confirming that TGF- β is deeply involved in controlling MPM proliferation. Further the confirmation of our results was the evidence of the significant inhibition of PCNA expression, an antigen expressed in the nucleus during the DNA synthesis phase whose expression is directly proportional to the degree of cell proliferation [29], in TGF- β -silenced MPM cells.

Moreover, we used a strategy based on the siRNA-mediated silencing of TGF- β to examine the consequences of TGF- β inhibition in MPM patient-derived cell lines representing all the three different MPM histological subtypes (epithelioid, sarcomatoid, biphasic): data showed both significantly inhibited cell proliferation and the strong downregulation of PCNA expression at the mRNA level in all tested MPM cell lines, although it was slightly different in each histotype, with the strongest reduction in PCNA expression observed in biphasic MPM cells, again underlining differential histotype sensitivity.

Based on the data obtained, we performed experiments to investigate the effect of TGF- β on both cell proliferation and cell cycle. After the inhibition of TGF- β , MPM cells accumulated in the S phase. This accumulation indicates that a critical checkpoint was impaired, specifically the transition from the S to the G2/M phase, thereby halting cell cycle progression. Conversely, the administration of TGF- β to the cell cultures resulted in a significant increase in the percentage of cells in the G2/M phase and a corresponding reduction in the G0/G1 phase. This unexpected G2/M arrest suggests that, in these specific cells, the TGF- β pathway may be involved in a different mechanism than its typical role in promoting G0/G1 arrest, with potential implications for the development of personalized therapies.

In our MPM cellular models, and in line with previous work from our research group [24], we observed that TGF- β can induce EMT in non-transformed human mesothelial cells, leading to increased motility, invasiveness, and resistance to apoptosis, as also reported in the literature [29]. Our research group has previously demonstrated that EMT plays a significant role in MPM. Given the established involvement of EMT in TGF- β -mediated effects in MPM, and considering its role in promoting invasiveness and metastasis, we investigated the migratory capacity of MPM cells following TGF- β silencing. Our results showed a significant reduction in cell migration after siTGF- β treatment, confirming that TGF- β is a key driver of motility in MPM and that its inhibition can impair the metastatic and aggressive potential of these tumor cells, likely via EMT suppression.

To further explore this mechanism, we assessed the expression of E-cadherin, a hallmark epithelial marker of EMT, following TGF- β silencing and/or treatment with Fresolimumab, a TGF- β -neutralizing antibody drug. We observed an upregulation of E-cadherin mRNA levels upon TGF- β inhibition, suggesting a partial reversal of the EMT process. These findings were corroborated by immunofluorescence analysis, which confirmed the increase in E-cadherin expression and indicated EMT reversion following TGF- β blockade. In parallel, the evaluation of fibronectin, a mesenchymal EMT marker, further supported this reversal, particularly after TGF- β silencing. Remarkably, siRNA-induced suppression proved to be more pronounced than antibody-mediated neutralization, likely due to its direct targeting of TGF- β mRNA, as opposed to the indirect action on the protein level exerted by the antibody. Taken together, these data demonstrate that targeting TGF- β in MPM attenuates EMT and reduces cell migration in vitro, supporting the potential of

TGF- β inhibition as a strategy to limit tumor invasiveness and improve therapeutic outcomes in MPM.

Finally, we assessed the effects of TGF- β blockade in more complex and physiologically relevant models by employing 3D spheroid cultures of MPM cells. These models, as recently highlighted in the literature, provide a more accurate representation of the mesothelioma tumor microenvironment compared to 2D cultures [30–32]. Treatment with the anti-TGF- β antibody or Fresolimumab significantly reduced spheroid growth compared to untreated spheroids, with growth rates reduced by more than half. These results corroborate and extend the anti-proliferative effects observed in 2D cultures, reinforcing the potential of TGF- β inhibition in MPM. Importantly, the reduction in spheroid growth occurred without evidence of cell death, suggesting a cytostatic rather than cytotoxic effect. This interpretation is supported by our cell cycle analysis, which showed arrest in the G1/G0 or S phases, indicating impaired proliferation rather than the induction of apoptosis. Furthermore, the lack of a significant increase in MTT results in treated spheroids compared to untreated ones, corroborating our interpretation. These findings highlight the ability of TGF- β inhibition to restrain tumor growth through a cell cycle blockade. Collectively, these results support the potential of TGF- β inhibition as a tumor growth-controlling strategy in MPM, particularly when considered in combination with cytotoxic agents to maximize therapeutic efficacy [33].

So, concerning the therapeutic management of MPM, it is crucial to acknowledge that this thoracic malignancy remains difficult to treat and often develops resistance to current therapies [8,34]. Several clinical trials have investigated the role of TGF- β inhibition in cancer, including MPM (*ClinicalTrials.gov* ID: NCT01112293, NCT02250846 and NCT02470894), aiming to assess its therapeutic potential and safety profile. But, while these studies showed promising preclinical data, the clinical trial data were not as encouraging, and additional studies were needed to optimize delivery and treatment protocols. Particularly, combination therapies are increasingly recognized as essential for improving MPM treatment outcomes. These studies primarily focus on monoclonal antibodies, small-molecule inhibitors, and TGF- β receptor antagonists.

In our study, we demonstrated the comparative efficacy of two distinct TGF- β inhibition strategies across multiple MPM cell lines, providing preclinical evidence of how TGF- β blockade influences key tumor cell functions, such as proliferation and invasion, particularly within the context of EMT mechanism, through which MPM metastasis may be attenuated. So, our findings may be supported by previous preclinical and clinical studies, particularly regarding enhanced therapeutic efficacy and a better understanding of cell line-specific responses, both of which could contribute to improve MPM management.

In the era of immunotherapy, the combination of TGF- β inhibitors with immune checkpoint inhibitors has emerged as a promising strategy [35]. However, clinical responses have been observed only in a subset of patients, underscoring the necessity to return to preclinical research. In-depth molecular and functional analyses of patients treated with TGF- β inhibitors are needed to better stratify individuals who may benefit from therapies targeting the TGF- β pathway [23].

Given the dismal prognosis of MPM, there is an urgent need to identify novel therapeutic strategies that can at least improve clinical outcomes. In this context, targeting TGF- β to suppress tumor proliferation and invasiveness represents a key research challenge. This approach aims to provide new mechanistic and preclinical in vitro evidence that supports a rationale for future in vivo validation, aligns with the broader goal of developing personalized medicine strategies aimed at improving survival, and offers more effective treatment options for patients displaying this aggressive cancer.

4. Materials and Methods

4.1. Cell Cultures

Experiments were performed on the commercially available MPM cell line MSTO-211H (human biphasic mesothelioma cells), purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and primary MPM patient-derived cell lines MM404 (human epithelioid histotype), MM421 (human biphasic histotype) and MM432 (human sarcomatoid histotype) from Biological Bank of Mesothelioma (SS. Antonio and Biagio and Cesare Arrigo Hospital, Alessandria, Italy), in accordance with the Institutional guidelines and according to the declaration of human ethics already approved (Protocol number #128/2016). All cells were grown in HAM's F12 or RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The cells were maintained at 37 °C in a humid atmosphere of 5% CO₂ and 95% air.

Some 24–48 h from seeding, cells were treated and analyzed as shown below.

4.2. SiRNA TGF- β Transfection

SiRNA targeting TGF- β 1/2/3 (siTGF- β) was purchased by Santa Cruz Biotechnology (sc-44146; Santa Cruz Biotechnology, Dallas, TX, USA). After 24h MPM cells, at 50% confluency, transfection was performed with jetPRIME[®] transfection reagent (Polyplus transfection, Illkirch, France) and siTGF- β (20 nM). MPM cells were treated at 37 °C for 48 h, then cells were processed as described below.

4.3. Anti-TGF- β Antibody or Fresolimumab Drug Incubation

Cells were treated with anti-TGF- β antibody or the Fresolimumab drug, a humanized monoclonal antibody against TGF- β which neutralizes all active subtypes of TGF- β . Anti-TGF- β antibody was purchased from Abcam (Cambridge, UK), and Fresolimumab was purchased from ProSci Incorporated (San Diego, CA, USA). In our experimental conditions, anti-TGF- β antibody and Fresolimumab were used at a concentration of 14 nM. Fresolimumab treatment was performed as described by Chopra A., 2004 [35].

4.4. Migration Assay

SiTGF- β -transfected and/or Fresolimumab-incubated cells grew in medium, until confluence. A scratch wound was generated with a pipette tip. Pictures were captured of each well immediately (T0) and after 24 h (T1) using a Leica DM IRB camera (Leica Microsystems, Milan, Italy). Images were analyzed using Leica LASX Software (version 3.1 Leica Microsystem). The relative migration rate was calculated by setting the percentage of migration of the control cells at time T1 and comparing the percentage of migration of the treated cells to this value. The results were representative of three independent experiments.

4.5. MTT Assay

The effect of proliferation of the treatments was assessed using the MTT assay kit. After seeding in 96-well plates, cells were transfected with TGF- β siRNA and or incubated with Fresolimumab for 48 h in a humidified CO₂ incubator at 37 °C. Next, 100 μ L of MTT reagent (0.5 mg/mL in PBS) was added to each well and after 4 h of incubation at 37 °C, the formazan crystals were solubilized by adding 100 μ L of DMSO. After incubation at 37 °C for 15 min, the absorbance was detected spectrophotometrically at 570 nm by a Synergy HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

4.6. Cell Cycle Analysis

Untreated and treated cells were detached and washed in PBS, permeabilized in 70% ethanol for 10 min and resuspended in PBS containing 0.1% (wt/vol) sodium citrate, 0.4

mg/mL DNase-free RNase-A, 0.1% (vol/vol) Triton X-100, and 50 µg/mL PI. Cellular DNA content was analyzed by FACS (Guava easyCyte™, Millipore, Burlington, MA, USA), measuring the cellular PI intensity. Histograms are representative of a double-peak distribution of cellular PI intensity. Analysis data was performed with the Software GuavaSoft™ (Version 3.1.1).

4.7. TGF-β Secretion Evaluation by ELISA

After treatment, the extracellular medium was collected. To determine the concentration of TGF-β in the supernatant, ELISA was performed according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA). Absorbance was measured at 450 nm with a Synergy HT microplate reader. The results were expressed as pg/mL of protein.

4.8. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was obtained by the guanidinium thiocyanate–phenol–chloroform method, using RiboZol RNA Extraction Reagents (Amresco, Solon, OH, USA), iScript cDNA Synthesis Kit (Bio-Rad Laboratories AG, Cressier, Switzerland) and IQ™ SYBR Green Supermix (Bio-Rad Laboratories AG), according to the manufacturer's instructions. PCR amplification was performed by CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories AG), as follows: 1 cycle of denaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and synthesis at 72 °C for 30 sec. The relative expression of each target gene was determined using CFX Maestro Software 2.3 (Bio-Rad Laboratories AG).

PCR specific primers:

S14: F 5' AGGTGCAAGGAGCTGGGTAT 3', R 5' TCCAGGGGTCTTGGTCCTATTT 3';

TGF-β: F 5' GTTGTGCGGCAGTGGTTGAG 3', R 5' GCCGGTAGTGAACCCGTG 3';

TGF-βR1: F 5' GAGGAAAGTGGCGGGGAGAA 3', R 5' ACACCAACCAGCTGAG-TCC 3';

TGF-βR2: F 5' TCTATGACGAGCAGCGGGGT 3', R 5' CTCAGTGGATGGGCAG-TCCT-3';

SMAD2: F 5' CCAATCGCCCATTCCCCTCT 3', R 5' GCCACGCTAG-GAAACAGCC 3';

SMAD3: F 5' GCCTGCTGGGCTGGAA 3', R 5' GATGGGACACCTGCAACCG 3';

E-cadherin: F 5' TACGCCTGGGACTCCACCTA 3', R 5' CCAGAAAC-GGAGGCCTGAT 3';

PCNA: F 5' ACCGGTTACTGAGGGCGAGA 3', R 5' AGGCGGGAAGGAG-GAAAGTC 3'.

4.9. Immunocytochemical Reactions: Fluorescence Microscopy Evaluation

Control and treated cells grown on sterile glass coverslips were fixed with 4% formalin for 10 min and washed for 2 min in PBS. The permeabilization step was executed for 5 min 1% Triton X-100 in PBS at room temperature, and the next wash step occurred in PBS. Then, unspecific sites were blocked using a blocking solution of PBS supplemented with 5% BSA in PBS for 30 min at room temperature. Next, cells were immunolabeled with primary antibodies diluted in 1% BSA in PBS overnight at 4 °C. After 3 washes in PBS of 5 min each, coverslips were incubated with secondary antibodies in diluted in 1% BSA in PBS (2 µg/mL, Goat Anti-Rabbit IgG-CF™488A Conjugate, Sigma-Aldrich, Milano, Italy; Goat Anti-Mouse IgG-TRITC Conjugate, Novex, Life Technologies, Carlsbad, CA, USA) for 1 h. At the end of the incubation and after other washing in PBS, sections were counterstained for DNA with 2 µg/mL DAPI (Sigma-Aldrich, Milano, Italy), washed with PBS, and mounted in a drop of Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich,

Milan, Italy), for fluorescence microscopy analysis. A Leica DM IRB camera (Leica Microsystems, Milan, Italy). Images were recorded and processed using Leica LASX Software. The fluorescence intensity was analyzed with the ImageJ software (<http://rsb.info.nih.gov/ij/>, accessed on 22 March 2021).

4.10. Spheroids Formation and Dimensional Analysis

Previously, siTGF- β -transfected and non-transfected cells were seeded in ultra-low-attachment (ULA) plates at a cell density of 3000 cells/well to obtain spheroids. After 48 h of formation, we treated the spheroids with or without Fresolimumab and monitored the size in the next 72 h. Pictures were captured of each well immediately (T0), after 24 h (T1), 48 h (T2) and 72 h (T3) using a Leica DM IRB camera (Leica Microsystems, Milan, Italy). Images were analyzed using Leica LASX Software. The results were representative of three independent experiments.

4.11. Statistical Analysis

Experiments were repeated three times. Statistical analysis of the results was performed by a one-way analysis of variance (ANOVA) and Tukey test using GraphPad Prism software (Version 9.5.1, San Diego, CA, USA).

5. Conclusions

Overall, although several aspects of the molecular mechanisms underlying MPM development and metastasis remain to be elucidated, our findings support the notion that TGF- β represents both a promising prognostic marker and a viable therapeutic target in the treatment of MPM, yielding mechanistic and preclinical in vitro evidence that provides a rationale for future in vivo validation in the attempt to be of use in a therapeutic approach. Thus, targeting TGF- β may offer a valuable strategy to combat this highly aggressive tumor, with the potential to improve patient prognosis and enhance pharmacological management. This approach becomes even more critical considering the expected rise in MPM incidence in these years.

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