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ORIGINAL ARTICLE

Turning back the Wheel: Inducing Mesenchymal to Epithelial Transition via Wilms Tumor 1 Knockdown in Human Mesothelioma Cell Lines to Influence Proliferation, Invasiveness, and Chemotaxis

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Abstract Malignant pleural mesothelioma (MPM) is a highly aggressive tumor that arises from the surface of the pleura and is associated with a history of asbestos exposure. The tumor is characterized by a strong local invasiveness and a poor response to any single modality therapy. Therefore clinical outcome of patients with MPM is poor and median survival time of untreated patients with MPM is 7 months from initial diagnosis. The Wilms Tumor Protein 1 (WT1) is a transcription factor which is highly expressed by MPM and is involved in cellular development and survival. We evaluated the role of WT1 in two human MPM cell lines (MSTO and H2052) expressing high levels of WT1. We performed a knockdown of WT1 using siRNA. Knockdown of WT1 was confirmed by Westernblotting. After knockdown of WT1 we investigated the effect on proliferation, chemoresistance, chemotaxis and migration. We could demonstrate that knockdown of WT1 suppresses chemoresistance in both cell lines compared with control (scrambled siRNA). Additionally, WT1 knockdown reduces proliferation, chemotaxis and invasiveness of mesothelioma cell lines. WT1 reduces malignancy of malignant mesothelioma cell lines and might be a new molecular target in mesothelioma therapy. Further investigations are needed to discover the mechanisms of chemoresistance depending on WT1.

Keywords Wilms tumor · Pleural · Asbestos · Pleuropneumonectomy · Decortication · Met · Stem cells · EPP · P/D · Epigenetics · EMT · Epithelial to mesenchymal transition · Cancer · Chemoresistance · Cisplatin · Drug

Introduction

Malignant pleural mesothelioma (MPM) is a rare malignancy with a median overall survival of 7 months [1] which is increased by therapeutic approaches only a few months [2]. Therapeutic options include radiation, chemotherapy and surgery mostly in a multimodal approach. However, due to the extreme resistance of MPM to common chemotherapies, new targets for therapeutic use are urgently needed. MPM mainly exists in three different histological subtypes, which are associated with different therapeutic response and clinical outcome [3]. The three different histological main subtypes are epitheloid, biphasic and sarcomatoid and may reflect three different grades on the axis of epithelial-to-mesenchymal transition (EMT) and its reverse process mesenchymal-to-epithelial transition (MET) [4]. EMT is a crucial process enabling cells to gain new capabilities like invading tissue and migrate to distant regions [5]. Wilms Tumor Protein 1 (WT1) is a transcription factor which is highly expressed by MPM [6] and is supposed to be a key regulator of EMT [7]. Interestingly, WT1 regulates also this axis backwards and is

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able to induce mesenchymal–to-epithelial transition (MET), leading to a re-differentiation [8, 9]. Although WT1 is widely accepted as a tumor suppressor in Wilms tumor [10], some authors demonstrated the pro-oncogenic role of WT1 in different malignancies [11–13]. However, the role of WT1 in MPM is still unknown. The aim of this study was to evaluate the role of WT1 in proliferation, chemotaxis, invasion, and chemo resistance in human MPM cell lines.

Material and Methods

Cell Culture

Human mesothelioma cell lines NCI-H2052 and MSTO-211H (ATCC, CLR-5915 and CRL-2081) were cultured in RPMI-1640 media with 10% fetal bovine serum, 100 μg/ml Streptomycin and 100 units/ml penicillin at 37°C in a humidified 5% CO_2 atmosphere. Cultures of cells were harvested at 80% confluence 24 h before transfection experiments.

Transfection Experiments

Pre-designed siRNA to WT1 were purchased from Santa Cruz Biotechnology, Dallas, USA) and applied according to the manufacture's protocol. Cells transfected with control-siRNA A (Santa Cruz Biotechnology) were used as controls. In all experiments, the effects of WT1 knockdown were assessed 72 h after siRNA transfection. All siRNA silencing experiments were performed in triplicates. Untreated cells are defined as "control", cells transfected with scrambled siRNA are defined as "scr" and cells transfected with WT1 specific siRNA as "WT1".

Reverse Transcription PCR (RT-PCR)

Total RNA was isolated using RNeasy Mini Kit (Qiagene, Düsseldorf, FRG). Reverse transcription was performed using iScript Kit (Bio-Rad, München, FRG). Specific primers for human WT1 and GAPDH were designed using AmplifX 1.5.4 by Nicolas Jullien; CNRS, Université Aix-Marseille (http://crn2m.univ-mrs.fr/pub/amplifx-dist) using NLM GenBank databases (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/nuccore/). Accession code numbers for the nucleotide sequences used to generate the respective primers and the primer sequences are depicted in supplemental Table 1.

All primers were intron-spanning and synthesized by Biomers (Biomers.net, Ulm, FRG). In case of WT1 the primers were designed to disregard differences of the various transcript variants of WT1. Real time PCR was performed using iQ SYBR Green SuperMix, iCycler thermocycler, and iCycler iQ 3.0 software (Bio-Rad Laboratories GmbH, München, FRG) according to the manufacturer's protocol. To control for specificity of the amplification products, a melting curve analysis was performed. No amplification of nonspecific products was observed in any of the reactions. A threshold cycle value (Ct) was calculated and used to compute the relative level of specific mRNA in our samples by the following formula:

$$\text{Brelative expression}\hat{} = 2^{(\text{Ct GAPDH-Ct target})} \times 10,000.$$

Western Blot

Cells were washed three times with ice-cold PBS, scraped from the bottom of the cell culture flask and lysed with lysis buffer (50 mM Tris-HCl pH 7,6, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0,1% Trition-X) containing a protease inhibitor cocktail. Total protein concentration was measured using BCA protein assay kit with bovine serum albumin as standard. Whole cell lysates were boiled at 93°C for 5 min in equal volumes of loading buffer (0,5 M Tris-HCl pH 6.8, 2% SDS, 0, 05% bromphenol-blue, 20% 2-mercaptoethanol, 10% Glycerol).

All samples were subjected to 12% sodium dodecylsulfate–PAGE, separated by electrophoresis and transferred to a polyvinylidene difluoride membrane (PVDF). After blocking for 2 h in Tris-buffered saline (TBS) containing 5% non-fat dry milk, the membranes were incubated with primary antibody diluted 1:2000 (WT1 antibody sc 192, Santa Cruz Biotechnology,) with TBS containing 5% non-fat dry milk at 4°C overnight. Visualization was performed using appropriate secondary antibodies labeled with 926–32,211 IRDye anti-Rabbit IgG (Li-COR Bioscience, Bad Homburg, FRG) diluted 1:20,000 for 2 h and scanned using Odyssey system (Li-COR Bioscience) according to the manufactures instructions.

Chemotaxis Assay

Before starting the assay all used cells were transfected as mentioned in the section "Transfection experiments". The effectiveness of each transfection was verified as mentioned above. Chemotaxis assays were performed using a 10-well chemotaxis chamber (NeuroProbe, Gaithersburg, U.S.A.). The bottom wells were filled with 400 μl of DMEM containing epidermal growth factor (EGF) in concentrations of 1 ng/ml. In parallel experiments, DMEM without chemokine was used as negative control. A 12 μm-pore-diameter polyvinylpyrrolidone-free polycarbonate filter (NeuroProbe, Gaithersburg, U.S.A.) was placed on the bottom plate. A silicon gasket and the top plate with 12 holes were then mounted, forming the top wells. 2×10^4 cells were added in a volume of 285 μl into the top wells. After 24 h of incubation at 37°C and 5% CO2, the filter sheet was removed, and non-

migrated cells were wiped off from the top side of the filter. The filter was then stained using crystal violet (Sigma-Aldrich, St. Louis, U.S.A.) and fixed in clear lacquer. Thereafter 9 high-power fields per filter were counted at 200-fold magnification using a Zeiss microscope.

Invasion Assay

Before starting the assay all cells used were transfected as mentioned in the section "Transfection experiments". The effectiveness of each transfection was verified as mentioned above. The ability of cells to migrate through a Matrigel basement membrane matrix was measured using the Cell Invasion Assay Kit (Chemicon International, Temecula CA, USA) with 8 μm membrane pore. After rehydration of the gels for 2 h, 1×10^5 cells in serum-free DMEM were applied to the upper chamber while the lower chamber contained EGF (1 ng/ml) in serum-free DMEM or serum-free DEMEM as control. After incubation at 37°C for 24 h, all cells of the upper chamber side were removed with a cotton swab. Subsequently, the membrane was removed and the invasive cells were stained using crystal violet. Analysis was performed by counting 9 high-power fields per filter at 200-fold magnification using an Olympus microscope (Olympus, Tokio, J.P.N.).

Chemoresistance Assay

Before starting the assay all used cells were transfected as mentioned in the section "Transfection experiments". The effectiveness of each transfection was verified as mentioned above. To measure the influence of WT1 on chemoresistance of the tumor cells, cells were cultured in a 6-well plate and grown to 80% confluence. Cells were treated with or without cisplatin at LD_{50} concentration as indicated for 72 h. Subsequently, a dilution series of cisplatin ranging from 0.4 to 25 μg/ml was added for additional 72 h. At the end of the assay period, living cells were measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay. The medium was changed and incubated with MTT solution (5 mg/ml in phosphate buffered saline)/ well for 4 h and the formazan developed during the reaction time was solubilized in isopropanol and measured photometrically at 563 nm. The optic density retrieved for non-treated cells was set to 100% and used to calculate the percentages of viable cells in the treated cultures.

BrdU -Proliferation Assay

Before starting the assay all used cells were transfected as mentioned in the section "Transfection experiments". The effectiveness of each transfection was verified as mentioned above. DNA synthesis was measured using a bromodeoxyuridine (BrdU) Cell Proliferation Kit (Calbiochem, Darmstadt, FRG). BrdU labeling solution was added to the cells in combination with the different treatments and incubated for 24 h. After removal of the culture medium, the cells were fixed, permeabilized and the DNA was denatured via micro wave. A peroxidase-labeled anti-BrdU antibody was then added for one hour. After rigid washing of the plate the signal was developed with tetramethylbenzidine solution in darkness. Absorbance in each well was measured using a spectrophotometric plate reader at 450 nm with a reference wavelength at 595 nm.

Statistical Analysis

Statistical analysis was conducted by using MedCalc software Version 11.6.1.0 (MedCalc software, Broekstraat 52, 9030 Mariakerke, Belgium). Values are shown as median and percentile of at least of a minimum of three independently conducted experiments. Comparisons of parameters were conducted using the analysis of variance (ANOVA). A $p < 0.05$ is considered as significant. All p values are Bonfferoni corrected. Only significant p values were included in the figures.

Results

Validation of siRNA Mediated Knockdown

Successful transfection of WT1 specific and scrambled siRNA was documented before each experiment via qPCR and Westernblotting. Effective silencing result in reduced expression of WT1 on mRNA *(*data not shown*)* and protein level (Fig. 1), whereas scrambled siRNA shows no effect on WT1 expression.

WT1 Knockdown Reduces Proliferation

We investigated the influence of WT1 knockdown on proliferation rate in a BRDU assay. After knockdown of WT1, proliferation was significantly reduced about a minimum of 25% in MSTO cells (control: 1.2, scr: 0.8, WT1: 0.6; $p < 0.02$, Fig. 2a) compared to scrambled siRNA and control MSTO cells. In H2052 reduction of proliferation was only significant between wild-type and WT1 specific knockdown with a reduction about 25% (control: 0.21, scr: 0.21, WT1: 0.16 $p < 0.04$, Fig. 2b). The difference of the proliferation rates between scrambled siRNA and WT1 specific siRNA was close to significance.

Fig. 1 Successful knockdown of WT1 verified on Westernblot. (lane 1: ladder, lane 2: WT1 wild type, lane 3 scrambled RNA, lane 4 and 5: knockdown with WT1-specific siRNA)

Effect of WT1 Knockdown on Chemotaxis and Invasion

Chemotaxis was clearly reduced in MSTO and H2052 cells after WT1 knockdown (Fig. 3a and b). Mean count in H2052 for chemotaxis: control 97 cells per view, scr 75 cells per view and WT1 39 cells per view. Mean count in MSTO: control 21 cells per view, scr 17 cells per view and WT1 12 cells per view. However, due to a large variation within the groups this reduction did not reach significance.

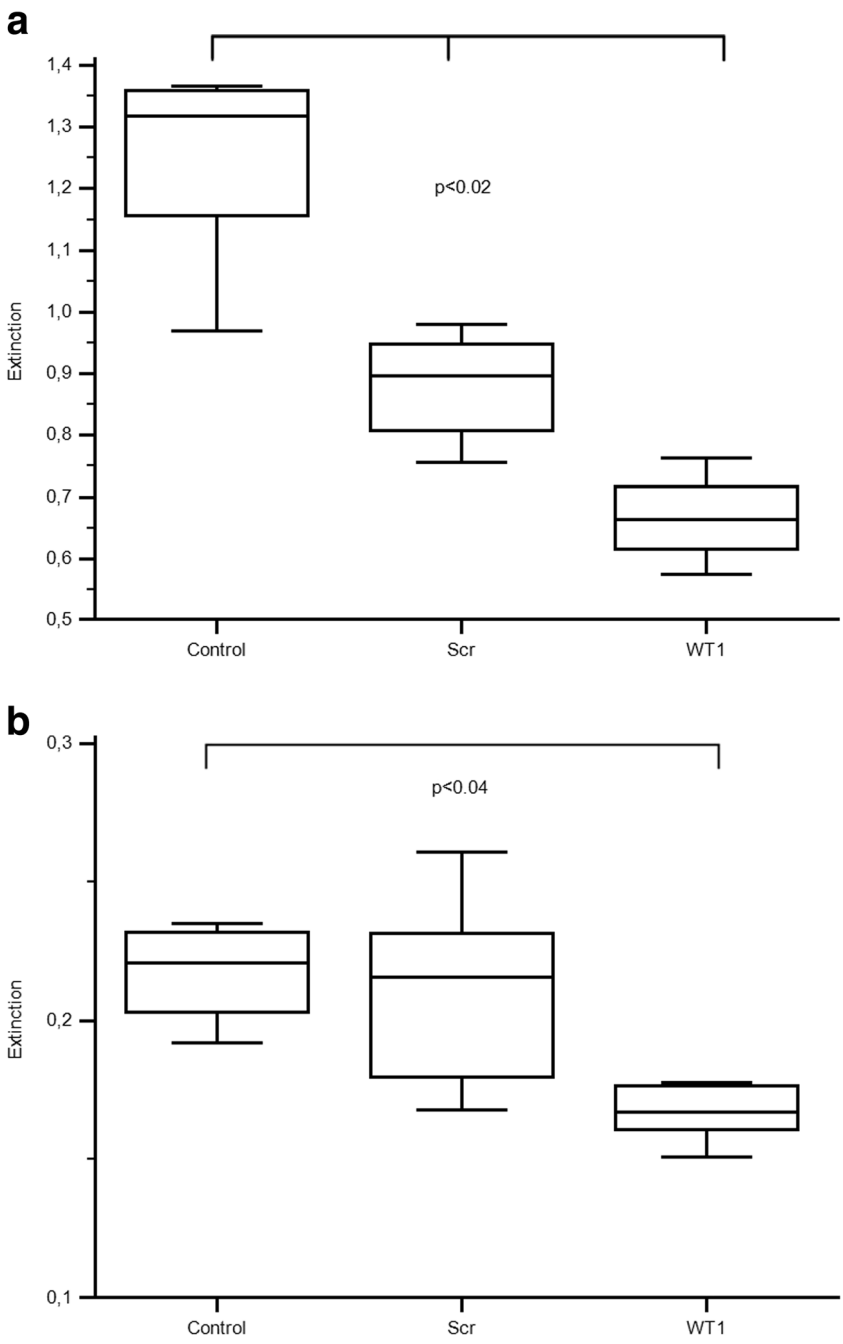

Fig. 2 WT1 knockdown reduces proliferation in MSTO (**a**) and H2052 (**b**) cells measured as BrdU incorporation. Data are given as optical density after detection of BrdU incorpoaration using a HRP-conjugated anti-BrdU-antibody (control: wild type cells, scr: scrambled siRNA, WT1: WT1 specific siRNA). In MSTO cells the reduction of proliferation was significant ($p < 0.02$ between each group of control, scr and WT1, p values were Bonferroni corrected) whereas the reduction in proliferation of H2052 cells did not reach significance

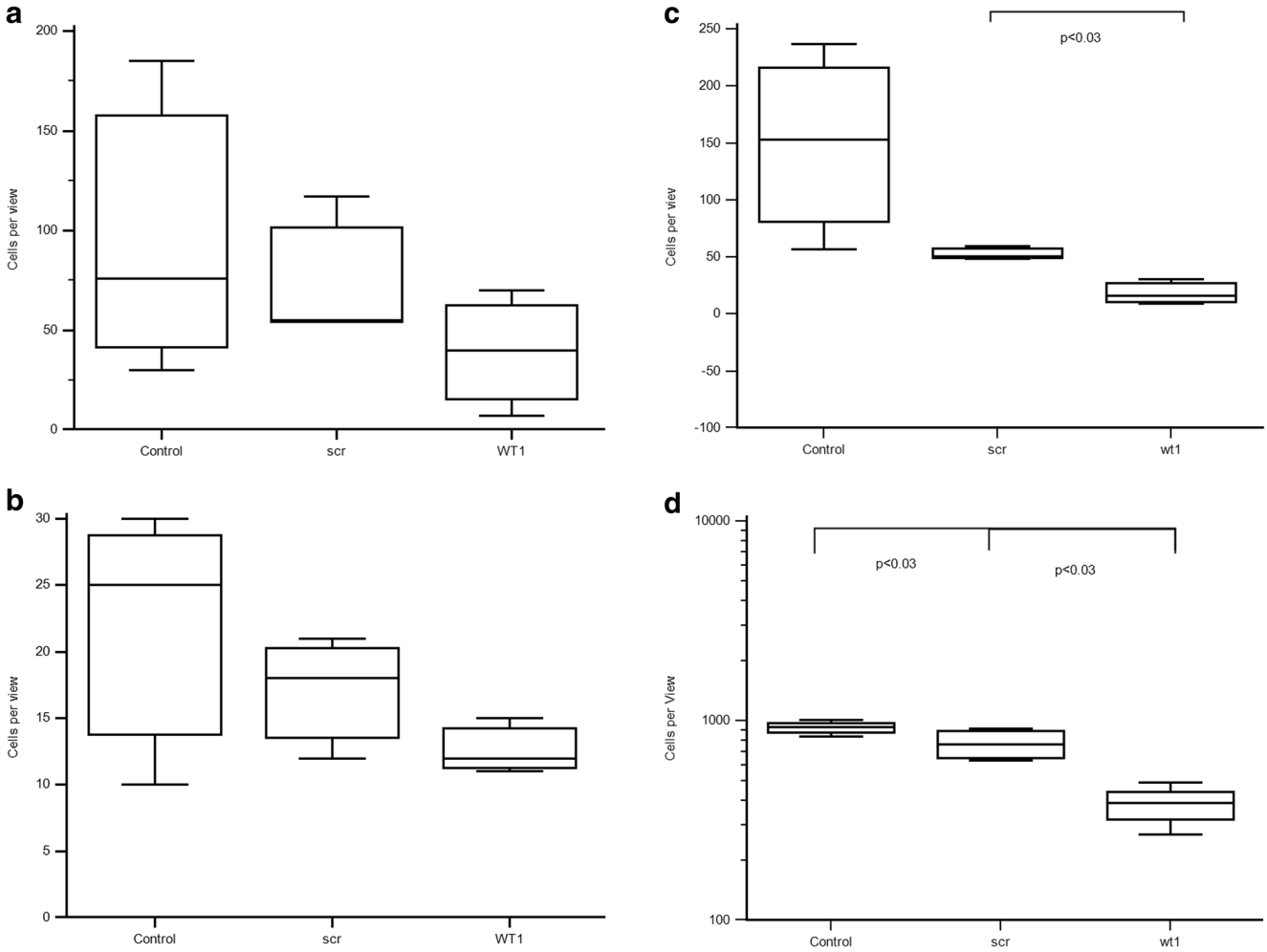

Fig. 3 Influence of WT1 knockdown (control: wild type cells, scr: scrambled siRNA, WT1: WT1 specific siRNA) on EGF-induced chemotaxis (a and b, not statistically significant) and epidermal growth factor (EGF) induced invasion (c and d, not statistically significant). WT1 knockdown reduced EGF-induced migration of H2052 cells (**a**) and of MSTO cells (**b**); however, these reductions did not reach significance. The invasion assay measures additional to migration also the capabilities of the cells to digest a matrigel basement membrane matrix. WT1 knockdown reduces significantly the invasive potential of both mesothelioma cell lines (c: H2052, ($p < 0.03$ between scr and WT1; not statistically significant: scr vs. control and control vs. WT1; p values were Bonferroni corrected); d: MSTO, $p < 0.03$ between control vs. WT1, scr vs. WT1; p values were Bonferroni corrected)

Invasion was also reduced after knockdown of WT1 and decreased dramatically ($p < 0.03$, Fig. 3 c and d) in both cell lines. WT1 knockdown reduces invasion in H2052 from 53 cells per view in scr to 18 cells per view in WT1 group. In control we found 149 cells per view. Using the cell line MSTO 926 cells per view were counted in the control group, 769 cells per view in scr and 383 in the WT1.

Chemoresistance Is Influenced by WT1 Knockdown

We investigated the resistance to cisplatin under the influence of WT1 knockdown. In both cell lines knockdown of WT1 decreased chemoresistance compared to the control and to wild-type cells. In MSTO cells, chemoresistance decreased from 0.71 in the control group to 0.66 in scr and 0.62 in WT1 ($p < 0.01$, Fig. 4 a and b). In H2052, chemoresistance increases from 0.93 in the control group to 0.97 in scr and decreases to 0.81 in WT1 (Fig. 4c and d). However, this reduction did not reach significance.

Limitations of the Study

The study has several limitations. It is unclear if the different histological subtypes of mesothelioma are influenced by epithelial-to mesenchymal transition. The siRNA knockdown also reduces WT1 expression only for a while. Therefore further investigations are needed to evaluate the role of WT1 in mesothelioma.

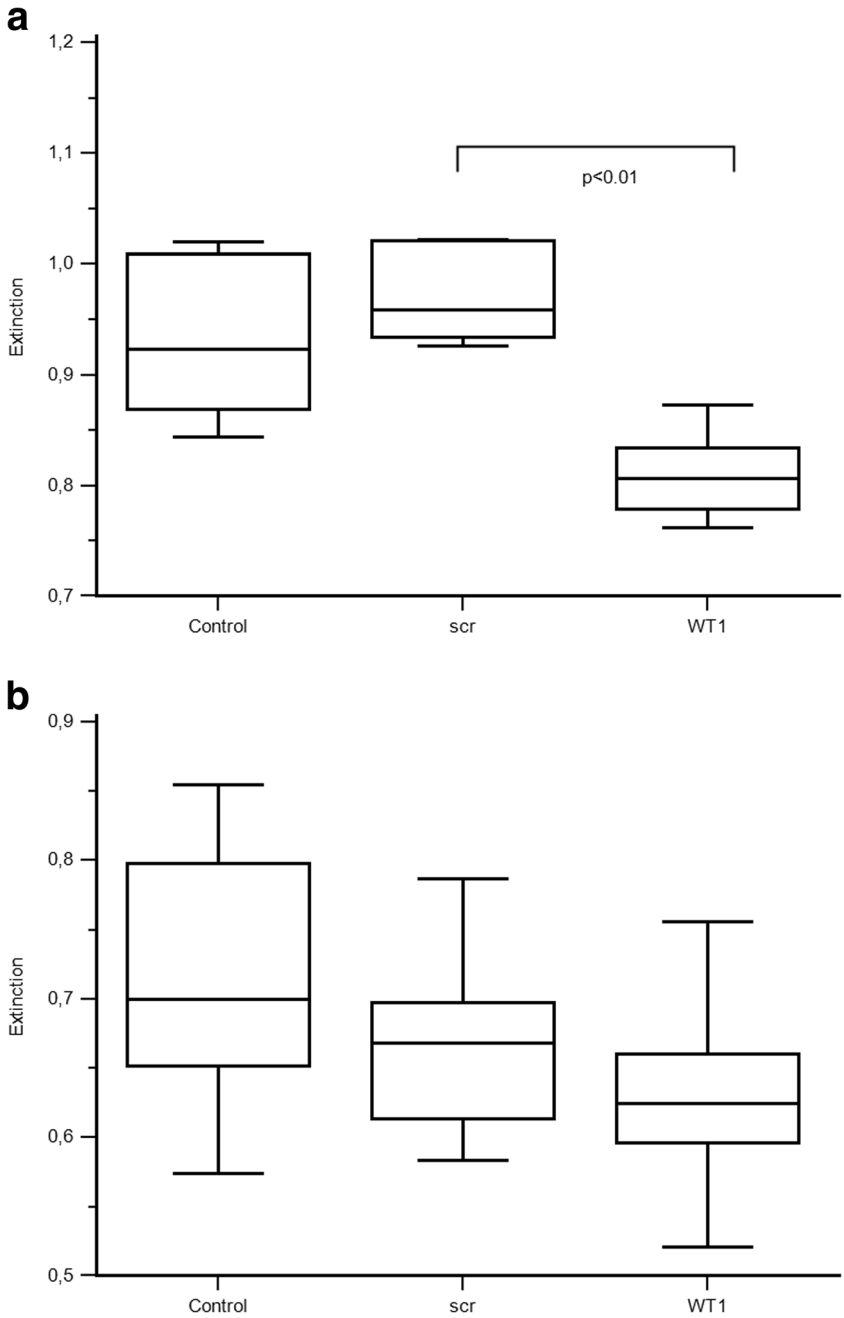

Fig. 4 Resistance to Cisplatin is reduced in mesothelioma cell lines (control: wild type cells, scr: scrambled siRNA, WT1: WT1 specific siRNA) if WT1 knockdown is performed. In MSTO cells (**a**), chemoresistance decreased from 0.71 in the control group to 0.66 in scr and 0.62 in WT1 ($p < 0.01$ for scr vs. WT1, not statistically significant for scr vs. control and control vs. WT1, p values were Bonferroni corrected). In H2052, chemoresistance increases from 0.93 in the control group to 0.97 in scr and decreases to 0.81 in WT1 (**b**). However, this reduction did not reach significance

Discussion

MPM is a rare disease, which is frustrating to treat [14]. Interestingly, it discloses remarkable features. Its extreme chemoresistance, its distinctive invasiveness and its aggressive growth combined with a low rate of metastasis in untreated patients make it interesting for further understanding of malignant growth. In epithelial carcinomas EMT is hypothesized to be a key process for malignancy and metastasis [15]. Based on the clinical observations that MPM treatment success on MPM is higher at the "epitheloid edge", the idea of this work was to push the phenotype of MPM cells back from mesenchymal to epitheloid phenotype in order to decrease aggressiveness and therapeutic response in ex-vivo systems employing cell lines. This is based on the fact that MPM is a tumor of mesenchymal origin implicating that MPM cells have undergone the process of EMT at least once. Little is known of the process of MET which is the reverse process of EMT, but at least MET may offer much more interesting approaches in identifying targets for treatment of diseases of mesenchymal origin. WT1 seems to be a key transcription factor connected to EMT as well as MET [16]. It has also a Janus-function, acting as proto-oncogene as well as a tumor suppressor [17]. Several WT1 isoforms have been identified,

but the functional differences between the WT1 isoforms are unknown. However, WT1 is highly expressed in MPM and we therefore investigated the role of WT1 in MPM concerning the most clinical relevant features of MPM like chemoresistance, proliferation and invasion. After knockdown of WT1 we observed a decreased proliferation rate. This is consistent with other studies, which also reported a reduced proliferation in broad spectra of malignancies after WT1 knockdown [18, 19]. The exact mechanism is still unknown, but WT1 may activate the STAT3 pathway, which leads to an enhanced proliferation rate [20]. Regarding that MPM is an aggressive growing tumor, WT1 may be an interesting therapeutic target in future therapeutic strategies. We also investigated the chemoresistance to cisplatin, which is a standard chemotherapeutic drug in MPM treatment. WT1 modulates several genes like BCL2, Cyclin D1 and interacts with p53 and Par-4 [21–23]. These genes are involved in proliferation, apoptosis and cell survival. Additionally high mRNA levels of WT1 are associated with chemoresistance in leukemia [24, 25]. Therefore WT1 may be also an interesting target for influencing drug resistance in MPM. Less surprising, both cell lines showed a massive chemoresistance to cisplatin. After knockdown of WT1 chemoresistance was notably reduced. A synergistic effect of WT1 knockdown with cisplatin was also documented in other malignancies [26, 27]. The aggressiveness of MPM manifests not only in its chemoresistance but also in the strong invasiveness of this entity. Local control is a key point where most therapy concepts fail in MPM treatment. We investigated the influence of WT1 knockdown on the capability of our cell lines to invade and observed that WT1 knockdown reduces the invasiveness dramatically. The same effect was already demonstrated in other tumor entities by several groups [28, 29]. Functional studies on WT1 suggest that it plays an active part in invasion, even if its exact mechanism remains unclear. WT1 plays various roles in ß-catenin signaling [30, 31] and promotion of cell adhesion via α-4 integrin is proved [29, 32].

We conclude that WT1 plays a key role in several features with relation to MPM treatment. WT1 may be an interesting target in this malignancy, which withstands most oncological treatment. New drugs are urgently needed to develop new treatment strategies in fight against MPM.

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