Cancer stem cells and epithelial-to-mesenchymal transition in lung cancer
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Chapter 3

TGF-β-induced Epithelial to Mesenchymal transition in A549 leads to enhanced chemoresistance, invasion and cancer stem cell properties

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Abstract

TGF-β is a potent inducer of Epithelial to Mesenchymal transition (EMT) in different tumor types, including non-small cell lung cancer (NSCLC). The acquisition of mesenchymal properties of tumor cells has been associated with an enhanced migratory and invasive capacity. In this study we used the TGF-β-inducible NSCLC A549 EMT model to study the possible effects of EMT on chemosensitivity, migration potential, invasive capacity and cancer stem cell properties. TGF-β-induced EMT in A549 cells was confirmed by the loss of epithelial markers EpCAM and E-cadherin and the gain of the mesenchymal markers Fibronectin and Vimentin. In cytotoxicity assays mesenchymal A549 cells were more resistant to cisplatin and showed enhanced migration and invasion potential as assessed by wound healing and transwell assays. Furthermore, mesenchymal A549 cells demonstrated increased spheroid forming potential in serum-free medium that coincided with enhanced expression of the known stem cell markers OCT4 and SOX2. Finally, an orthotopic mouse model was set up to explore the metastatic spread of transpleurally injected parental and TGF-β-induced mesenchymal luciferase-transfected A549 cells (A549-luc). Our preliminary comparative experiments detected high levels of metastatic spread in A549-luc injected cells that was not enhanced by pretreatment with TGF-β. In conclusion, our data indicate that mesenchymal A549 cells are more aggressive than their epithelial counterparts as demonstrated by the increased chemoresistance, higher migration and invasion potential and enhanced CSC properties in vitro.
Introduction

Lung cancer is the leading cause of cancer related deaths worldwide, being 28% of all male and 26% of all female death [1]. According to cancer statistics 2014, the estimated new cases of lung cancer in the USA of males and females are 116,000 and 108,210 respectively, which is second highest after prostate and breast cancer [1]. Histologically lung cancer is divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC attributes to 85-90% of lung cancer cases and is characterized with a high rate of metastasis and relative resistance to chemotherapy and radiotherapy. Surgical resection of the tumor is considered the best option for NSCLC treatment, which can ultimately be achieved in approximately 25% of cases only, due to the usually late clinical presentation of patients [2]. Usually, at time of diagnosis the tumor has already metastasized and the efficacy of applied chemotherapy is limited leading to 5-year survival rates of less than 5%. Recent advances in therapy such as the use of EGFR and ALK inhibitors have improved these five-year survival rates by 4-5% [3].

Epithelial to Mesenchymal transition (EMT) is a physiological process occurring during early embryogenesis and wound healing as well as in the metastatic spread of malignant cells in order to facilitate cell migration [4]. Hallmarks of EMT are loss of E-cadherin expression, loss of cell-cell junctions and cytoskeletal reorganization leading to the acquisition of a mesenchymal phenotype [5]. In cancer EMT has been implicated to facilitate the detachment of tumor cells from the primary site to subsequently enter the invasion- metastasis cascade. In this process intravasation of tumor cells takes place from primary sites to the blood or lymphatic circulation. At distant sites tumor cells can extravagate and colonize tissues resulting in tumor micrometastasis that can develop to macroscopic metastatic lesions [6]. Furthermore, activation of EMT in cells has been associated with the enrichment of cancer stem cell (CSC) properties [7]. Several transcription factors are involved in regulating EMT such as SNAIL, SLUG, TWIST and ZEB1 by modulating the expression of specific genes including the repression of E-cadherin [4]. EMT transcription factors are also controlled by external stimuli coming from the microenvironment such as FGF, IGF, EGF and TGF-β [8]. TGF-β regulates several biological processes that are important in cancer progression such as proliferation, apoptosis, and angiogenesis and is a major extracellular factor regulating EMT in vitro and in vivo [9,10].

In the NSCLC A549 cells treatment with TGF-β is known to potently induce EMT that is characterized by downregulation of epithelial markers such as E-cadherin and cytokeratins and upregulation of mesenchymal markers Fibronectin and Vimentin
Induction of EMT has been previously associated with enhanced chemoresistance and anti-apoptotic phenotypes in solid tumors [8].

In the present study we used the TGF-β-inducible A549EMT model to explore the involvement of EMT in chemosensitivity, migration, invasion and CSC properties. Further, we employed a luciferase transfected A549 orthotopic mouse model (A549-luc) to evaluate tumor growth and metastatic spread of epithelial and mesenchymal A549 cells and also attempted to detect circulating tumor cells (CTCs) in the blood of these mice using Bioluminescent imaging (BLI).

**Material and Methods**

**Cell lines, cell culture and chemicals**

NSCLC A549 cells (American Type of Culture Collection, ATCC, Manassas, USA) and A549-luc cells (PerkinElmer, Waltham, USA) were cultured as monolayers in RPMI-1640 medium (Life Technologies, Bleiswijk, The Netherlands) supplemented with 10% FCS (Bodinco, Haarlem, The Netherlands). Cells were maintained at 37°C in a humidified chamber supplied with 5% CO₂ and regularly tested for authenticity by short tandem repeats (STR) profiling and for mycoplasma infection. A549-luc cells were purchased from Caliper Life Sciences (Hopkinton, Ma, USA) and were maintained in a similar way.

A549 cells were treated with 10ng/ml of TGF-β for the indicated times (Peprotech, London, UK) to induce EMT. For spheroid culturing A549 cells were cultured in Neurobasal media (NBM) (Life Technologies, Bleiswijk, The Netherlands) supplied with 2% of B27 supplement (Life Technologies, Bleiswijk, The Netherlands), EGF 20ng/ml (R&D systems, Abingdon, UK) and b–FGF 10ng/ml (Millipore, Amsterdam, The Netherlands) in presence and absence of TGF-β. For prolonged culturing media was refreshed every 7 days.

When indicated cells were treated with a small molecule inhibitor of the TGF-β receptor, A83-01 (Axon Medchem, Groningen, The Netherlands). The inhibitor was added at a concentration of 0.5µM 4 hrs prior to the addition of TGF-β.

**Western blotting**

Western blot analysis was performed as described before [12]. To study EMT the following primary antibodies were used: mouse monoclonal E-cadherin 1:1000 (clone 36/E-cadherin, BD Biosciences, Breda, The Netherlands), mouse monoclonal
Fibronectin 1:1000 (clone 10/Fibronectin, BD Biosciences) and mouse monoclonal Vimentin 1:500 (clone sc-6260, Santa Cruz biotechnology, Bioconnect, Huissen, The Netherlands). To study CSC marker expression the following primary antibodies were used: rabbit polyclonal OCT4 1:1000 (ab19857, Abcam, Cambridge, UK) and mouse monoclonal SOX2 1:1000 (clone L1D6A2, Cell Signaling, Bioke, Leiden, The Netherlands). Equal loading of protein was confirmed by β-Actin staining using 1:10000 (clone C4, ICN Pharmaceuticals, Zoetermeer, The Netherlands). The membranes were incubated with primary antibodies overnight at 4°C and next day washed 3x for 5 minutes with Tris-buffered saline with and 0.1 % Tween-20 (TBST, pH 8.0) and incubated with the appropriate horseradish peroxidase (HRP)-labeled secondary antibody (all 1:1000, all from DAKO, Glostrup, Denmark) for 1 hr at room temperature. Proteins were visualized by chemiluminiscence using BM chemiluminiscence detection kit (Roche Applied Science, Almere, The Netherlands). Experiments were performed at least three times.

**Chemosensitivity assays**

For chemosensitivity testing A549 cells were seeded at 5,000 cells in 100µl RPMI+10% FCS medium per well in quadruplicate in 96-wells microtiter plates. TGF-β-treated cells were maintained in 10ng/ml TGF-β. After 24 hrs cells were incubated with increasing concentrations of cisplatin (Accord Healthcare BV, Rijsbergen, The Netherlands) in a total volume of 200µl for a further 4 days. Next, 20µl of 5 μg/µl MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to each well for 225 minutes. Plates were centrifuged and media (supernatant) was carefully removed from the wells and formazan crystals were dissolved in 200µl of DMSO. The absorbance was measured at 520 nm with an iMark Microplate Absorbance Reader (Bio-Rad laboratories, Veenendaal, The Netherlands). Controls consisted of medium without cells (background) and cells incubated with medium without drugs (growth control). At least three independent experiments were performed.

**Migration assays**

Cell migration potential was determined by using a wound-healing assay. For this, 50,000 A549 and A549+ TGF-β cells were seeded in 6-well plates for 72 hrs. Upon confluency a scratch was made at the center of the well using a 10µl pipette tip and after washing with PBS fresh media was added, with or without 10ng/ml TGF-β. Photographs of wound closure were taken at time point 0 hrs, 4 hrs, 8 hrs and 24 hrs with a light microscope at 10X magnification (Leica DM 3000 Rijswijk, The Netherlands).
Netherlands) and ‘wound closure’ was measured and analyzed by image J software (National Institute of Health, NIH, Bethesda USA). Experiments were performed three times.

**Invasion assays**

Cell invasion capacity was determined using Transwell chambers with 6.5mm, 8.0µm pore polycarbonate membrane inserts (3422, Corning, Amsterdam, The Netherlands). Inserts were coated with 30µg/ml of collagen type-1 (BD Biosciences) in PBS for 1 hr at 37°C. Collagen was removed and inserts were blocked with 1% BSA in PBS overnight at 37°C. A549 and A549+ TGF-β cells were trypsinized, and resuspended in low serum RPMI 1640 (RPMI-1640+ 0.1% FCS) and 50,000 of both types of cells were seeded in the upper chamber of the transwell. In the lower chamber RPMI-1640 supplied with 10% FCS was used as a chemoattractant. RPMI-1640+ 0.1% FCS was used as a negative control. After 6 hrs of incubation at 37°C, membranes were fixed in 75% methanol/15% acetic acid, and subsequently stained with coomassie-blue in 45% methanol/10% acetic acid for 10 minutes and invasive cells were quantified by microscopic counting. For each condition, cells in at least three independent fields were counted and the average was calculated. Experiments were performed for at least three times (and average of all experiments were plotted as mean+/− SD).

**Flow cytometry**

Cells were harvested and washed with FACS buffer (PBS at pH 7.2 with 0.5% BSA and 2mM EDTA) and pelleted by centrifugation. After resuspending cells in 100µl of FACS buffer, cells were stained with mouse monoclonal CD326 (EpCAM) -APC 1:10 (clone HEA-125, Miltenyi Biotech, Leiden, The Netherlands) and incubated on a rotary shaker in the dark for 30 minutes at 4°C. Corresponding isotype-matched antibodies directly labeled with APC and unstained cells were used as negative controls. After incubation cells were washed with cold FACS buffer, centrifuged and resuspended in FACS buffer for analysis by flow cytometry (BD FACSCalibur, BD Biosciences). Data were quantified and analyzed with WinList (Verity Software House, Topsham, USA).

**Cell sorting and spheroid formation assay**

A549 cells were grown in NBM with or without 10ng/ml TGF-β for five days and subsequently centrifuged, trypsinized, washed with PBS and resuspended in NBM. Cells were treated with DAPI (Life Technologies Bleiswijk, The Netherlands) for 5
minutes and processed for cell sorting using the MoFlo XDP (Beckman Coulter, Woerden, The Netherlands). Defined numbers of cells (10, 20, 50 and 100) were seeded in 96 well plates in triplicates with 100µl of either supplemented NBM or NBM+ 10ng/ml TGF-β. Plates were incubated for 7 days, and after adding 100µl additional fresh NBM or NBM+ TGF-β plates were incubated for a further 7 days. The number of spheroids identified as floating aggregates with approximately 3-5mm diameter in size was counted from each well using 10X magnification of light microscope. The spheroid forming potential was calculated as the average number of spheroids formed per number of seeded cells per well. Experiments were performed at least three times and average of all experiments were plotted as mean +/- SD).

A549-luc orthotopic mouse model
Studies in mice were approved by the institutional ethics committee for animal research of the University of Groningen and executed in accordance with the regulations of Dutch law on animal welfare. NOD-SCID IL2-γ mice (6-8 weeks) bred at the central animal facility of the institute were used. Animals were fed at libitum and kept under sterile conditions in ventilated cages. Animals were divided in two groups; the first group was injected with 0.5*10^6 epithelial A549-luc (n=4) and the second group with 0.5*10^6 mesenchymal A549-luc+ TGF-β cells (n=4). Cells were transpleurally injected in the lungs as described previously [13]. Briefly, animals were anaesthetized, rested on their right lateral decubitus, shaved at left scapula to make visible their chest skin. A small 2-cm long skin incision was made below the left scapula, a chest muscle was removed and costal layer and ribs were located. Tumor cells in a 1:1 solution containing of culture medium and matrigel (BD Biosciences) in a final volume of 20µl were directly injected through the intercostal space into the lung at a depth of 3mm. Skin was sutured and animals were recovered and placed in separate cages for the first week, followed by pair-wise housing. Tumor development and metastatic spread was determined once a week by BLI using Xenogen platform (PerkinElmer, Waltham, USA). For this mice were intraperitonially injected with 100ul; 150mg/kg D-luciferine K+ salt bioluminescence substrate (PerkinElmer, Waltham, USA) and the BLI signal was measured after 5 minutes.

For CTC detection, approximately 100µl of blood was collected in heparin containing vials by cheek puncture once weekly, starting two weeks after tumor cell injections. For quantification of CTC levels, BLI with increasing amounts of A549-luc cells were performed. Increasing amounts of previously generated PC-3 luc cells were used as
a positive control [14]. All animals were sacrificed at 7th weeks after orthotopic injections, when one animal from the A549-luc group showed difficulty in breathing and sudden loss of weight. All animals were subjected to a final BLI and peripheral blood (approximately 500µl) was collected through tail vein puncture for final CTC count. Animals were dissected and both lungs were recovered for BLI to measure primary tumor load and loco-regional invasion. For detection of distant metastatic spread liver, testis, kidneys, heart, brain, skin and ribs were recovered and their BLI signals were measured.

**Statistics**
All experiments were performed at least three times independently. Statistical analysis was performed for chemosensitivity, migration potential, invasive capacity, spheroid forming potential and for animal experiment to quantify primary and whole body tumor load by BLI using a double-sided, paired Student t-test. A p-value < 0.05 was considered significant.

**Results**

**TGF-β-induced EMT in A549 cells**
A549 cells were treated with 10ng/ml of TGF-β and after 72 hrs cells showed a stretched fibroblast-like phenotype and grew more dispersed than the untreated cells that formed cobblestone-like tightly packed confluent monolayers (Figure 1A). The growth rate of TGF-β treated cells was somewhat reduced after 2-3 days compared to untreated A549 cells and returned to normal at later time points (Supplementary figure 1). The mesenchymal-like phenotype of TGF-β-treated cells was further confirmed by determining the expression of different epithelial and mesenchymal markers. Flow cytometry analysis showed high expression of the cell surface epithelial marker EpCAM (CD326) in A549 cells that was strongly reduced in A549+TGF-β cells (Figure 1B). In addition, we also examined the effect of long term TGF-β exposure by maintaining these A549 EMT cells under a 5ng/ml TGF-β concentration for at least three passages and observed the same fibroblast-like morphology (not shown). However, culturing of these cells in the absence of TGF-β resulted in a gradual reversion (within next two passages) to an epithelial morphology, showing similar levels of EpCAM expression as parental A549 cells (not shown), indicating reversibility of EMT upon TGF-β removal. In western blot analysis A549 cells treated with TGF-β showed a time dependent decrease in expression of epithelial E-cadherin and increase in expression of the mesenchymal markers Fibronectin and Vimentin (Figure 1C). An almost complete loss of E-cadherin expression in A549 cells was
observed after 72 hrs of TGF-β treatment while Fibronectin and Vimentin expression was high indicative of a mesenchymal phenotype. This exposure time was used in the further experiments to obtain mesenchymal A549+ TGF-β cells. Furthermore, pretreatment of A549 cells with the small molecule inhibitor A83-01, a potent blocker of the TGF-β type I receptors ALK4, ALK5, and ALK7 effectively prevented TGF-β-induced EMT as indicated by the marker expression profile [15] (Figure 1D).

Figure 1: TGF-β induces EMT in A549 cells.
(A) Morphological differences between A549 and A549+ TGF-β treated cells the later showing a more elongated, spindle shaped and dispersed phenotype. (B) Cell surface expression of EpCAM as measured by flow cytometry in A549 and in A549+ TGF-β treated cells. A549 cells showed high expression of EpCAM compared to A549+ TGF-β treated cells. (C) Protein expression of E-cadherin, Fibronectin and Vimentin was determined by western blotting. A549+ TGF-β treated cells showed a time dependent downregulation of E-cadherin and upregulation of Fibronectin and Vimentin. (D) Protein expression of E-cadherin, Fibronectin and Vimentin in A549 cells and treatment with TGF-β in presence or absence of the inhibitor A83-01. Treatment of A83-01 abrogated the TGF-β induced EMT in A549 cells.

Mesenchymal A549 cells have enhanced chemoresistance, migration potential and invasion capacity

In order to examine possible differences in chemosensitivity we tested cisplatin sensitivity in A549 and A549+ TGF-β cells using the MTT assay. A549+ TGF-β cells were significantly more resistant (p=0.01) when compared to A549 cells (Figure 2A).
Next, the migration potential of A549 and A549+ TGF-β cells was examined by use of the wound-healing assay. Wound closure was determined at different time points (0, 4, 8 and 24 hrs) and A549+ TGF-β cells showed significant enhanced migration potential when compared to A549 cells at all time points (Figure 2B). Quantification of the results showed that A549+ TGF-β cells had an approximately two-fold higher migratory capacity when compared to A549 cells at 4 (p=0.05) and 8 hrs (p=0.007). At 24 hrs the scratch was almost closed in A549+ TGF-β cells, whereas in the untreated A549 cells the scratch was still visible (Figure 2B, C). The invasive capacity of A549 and A549+ TGF-β was tested using collagen coated transwell chambers. A549+ TGF-β cells displayed enhanced invasive capacity when compared to A549 cells (Figure 2D, E). The basal level of invasion (0.1%FCS) of A549+ TGF-β cells was approximately 4-fold higher than of parental A549 cells (p<0.001) and the invasive capacity was further enhanced in the presence of 10% FCS as chemoattractant (Figure 2D, E).

**TGF-β induced EMT enhanced CSC properties in A549 cells**

Next we addressed whether mesenchymal A549 cells may have altered CSC properties when compared to epithelial counterparts. For testing this we employed the spheroid formation assay by culturing the cells under serum-free supplemented NBM that previously in other studies was found to select for less differentiated cells having stem cell-like features [16]. Upon culturing cells in supplemented NBM for 14 days we noted that TGF-β exposed A549 cells had a higher spheroid forming capacity than their untreated epithelial counterpart that mostly formed adherent colonies and only few spheroid-like aggregates (Figure 3A). The spheroid forming potential of A549 and A549+ TGF-β cells was further determined by seeding different numbers of cells (10, 20, 50 and 100 cells). Cells grown in the presence of NBM+ TGF-β had a spheroid forming potential ranging approximately from 6-10% compared to around 3% in the A549 cells grown in the absence of TGF-β (Figure 3B). Spheroid forming potentials were significantly different for A549 and A549+ TGF-β cells at all cell densities (p=0.003, 0.008, 0.02 and p<0.001, for 10, 20, 50 and 100 cells per well, respectively). Furthermore, we also tested the expression of the CSC markers OCT4 and SOX2 under these two culture conditions by western blotting. A549 cells grown in supplemented NBM+ TGF-β showed clearly higher expression of OCT4 and SOX2 compared to the cells grown only in NBM (Figure 3C).

**Tumor growth and metastatic spread of A549-luc and A549-luc+ TGF-β cells in an orthotopic mouse model**

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We proceeded by exploring whether the differences in invasive potential and CSC properties observed \textit{in vitro} between A549 and A549+ TGF-\(\beta\) would also be relevant for tumor formation and metastatic spread in an \textit{in vivo} setting. Therefore, a pilot experiment was performed employing an orthotopic mouse model in which growth

![Graph showing Cisplatin sensitivity](image1)

![Wound healing assay results](image2)

![Transwell invasion assay results](image3)

**Figure 2: Cisplatin sensitivity, migration and invasion capacity in A549 and A549+ TGF-\(\beta\) cells.**

(A) Cisplatin sensitivity was measured by MTT assays showing significant higher resistance of A549+ TGF-\(\beta\) cells. (B) Migration potential of A549 and A549+ TGF-\(\beta\) cells was determined by wound healing assay. A549+ TGF-\(\beta\) cells showed enhanced wound closure activity as compared to A549 cells. (C) Quantified data of migration potential (D) Invasion capacity of A549 and A549+ TGF-\(\beta\) by transwell assays showed that A549+ TGF-\(\beta\) cells had higher invasive capacity then A549 cells. Representative pictures are shown. (E) Quantified data of invasion capacity. All the experiments were performed for at least three times and average of all experiments were plotted as mean+/- SD.
and metastatic spread of transpleural injected A549-luc cells could be studied, as previously described by Mordant et al [13]. First, following treatment of A549-luc cells with TGF-β for 72 hrs downregulation of E-cadherin and upregulation of Fibronectin and Vimentin were observed confirming similar EMT induction as earlier demonstrated in parental A549 cells (Figure 4A). Then A549-luc or TGF-β-treated A549-luc cells were transpleurally injected into the left lung of the NOD-SCID IL2-γ mice. Removal of the skin of the left flank of an animal and subsequent transpleural injection between the upper 2e and 3e ribs is shown in Figure 4B. One mouse in the untreated A549-luc group died within 2 weeks after injection, likely due to the surgical procedure.

Figure 3: TGF-β–treated A549 cells display enhanced CSC properties.
(A) Cell morphology of A549 cells grown in the absence or presence of TGF-β in NBM, indicated that TGF-β treatment greatly stimulated the spheroid growth of A549 cells. (B) Limiting dilution assays showing significantly enhanced spheroid forming potential of TGF-β exposed A549 cells. The mean +/- SD of three independent experiments is indicated. (C) Protein expression of OCT4 and SOX2 was increased in A549 cells grown in NBM+ TGF-β medium compared to cells grown in the absence of TGF-β.

All animals were monitored by BLI at different times post-injection and tumor growth in the left lung was detected in all animals (Figure 4C). In the animals a gradual increase in BLI signals upto week 6 post-injection was detected. At week 6, dorsal imaging showed metastatic spread to the right lung and in the thoracic cavity in all 4 animals of the A549-luc+ TGF-β group in addition to primary tumor growth in the left
lungs (Figure 4D). However, one mouse in the A549-luc group showed extensive tumor growth and distant metastasis in the left femoral bone that was not seen in the other two animals. In one animal from the A549-luc+ TGF-β group also metastasis to the left femoral bone was detected (Figure 4C, D).

Quantification of the BLI signal of the primary tumor and whole body tumor load in this pilot experiment was not possible due to the large variation in signal in combination with the small numbers of animals. Pathological examination of the animals confirmed massive tumor growth at the primary site and in the right lung. Metastatic lesions in the liver were also found in all animals. A representative photographic image and the corresponding BLI levels of the left lung (primary), right lung (loco-regional invasion) and liver (distant metastasis) are depicted from an A549-luc mouse (Figure 5A). Distant metastatic sites such as in kidneys, brain and adrenal glands were found by BLI in the resected organs (Figure 5B). Taken together, the A549-luc model allows the study of metastatic spread. In this pilot experiment no differences in tumor growth and spread could be detected between the A549-luc and A549-luc+ TGF-β injected animals.

An attempt was made to monitor the number of CTCs in the animals. Peripheral blood was collected biweekly starting from 2nd week post-injected animals for BLI measurements to detect the possible presence of CTCs. In vitro BLI measurements on different numbers of A549-luc cells showed the ability to detect around 50 tumor cells in suspension. However, we did not detect BLI signals in any of the blood samples of the animals, even not in a larger volume of peripheral blood (approximately 500µl) collected when animals were sacrificed (see supplementary Figure 2).

Discussion
In this study we employed the TGF-β inducible A549-EMT model to explore the effect of EMT on different tumorigenic properties such as chemosensitivity, migration potential, invasive capacity and CSC properties. After confirming TGF-β-induced EMT in A549 cells by showing morphological changes and loss of epithelial and gain of mesenchymal markers, we found that mesenchymal A549 cells have enhanced resistance for cisplatin a drug that is frequently used to treat NSCLC. Although TGF-β treatment induced a growth delay after 2-3 days, which may contribute to reduced sensitivity for cisplatin, at earlier and later time points cell growth was similar to untreated A549 cells. Cisplatin resistance may thus be associated with a
mesenchymal phenotype. Evidence for therapy resistance in cells undergoing EMT was recently provided by other researchers showing that modulation of the expression of specific microRNAs, including miRNA17 and miRNA10a, which both regulate TGF-β signaling can reverse cisplatin resistance in generated resistant A549

Figure 4: Metastatic spread and effect of TGF-β in the A549-luc orthotopic mouse model.
(A) TGF-β effectively induced EMT in A549-luc cells as demonstrated by western blotting. E-cadherin expression decreased and Fibronectin and Vimentin expression was enhanced by TGF-β exposure (72 hrs).
(B) Illustration of surgical procedure to expose the left site ribs (black arrow) and transpleural injection (white arrow) of the A549-luc cells. For details see methods section. (C) Tumor growth and metastatic spread was visualized by BLI. Animals injected with A549-luc and A549-luc+ TGF-β cells both showed time-dependent increases in BLI. One animal in the A549 control group succumbed in the experiment. (D) A ventral view of the animal at week-6 post injection.
Figure 5: Analysis of tumor growth and metastatic spread of A549-luc and A549-luc+ TGF-β cells. 
(A) Pathological analyses of tumor growth at the left lung (primary tumor) and metastatic lesions found in the adjacent lung and liver. Representative images obtained by BLI are shown. (B) Metastatic lesions were also detected in the left and right kidney (LK and RK), the brain and in the adrenal glands (AG) of some animals as displayed by BLI from a representative animal from A549-luc group.

Furthermore, knock down of the EMT transcription factors SNAIL or TWIST was earlier demonstrated to sensitize A549 cells to cisplatin treatment [19,20] and EMT in A549 cells also correlated with increased resistance to the EGFR inhibitors gefitinib and erlotinib [21].

TGF-β-induced EMT was found to enhance both the migration and invasion capacity of A549 cells in agreement with previous studies [11,22]. Increased migration and invasion potential upon EMT induction also has been reported in earlier studies. For example, EMT induced by ionizing radiation, involving activation of TGF-β signaling, displayed enhanced migration and invasion potential in lung cancer cell lines, including A549 [23].
In the current study we also attempted to evaluate the effect of EMT on tumor growth and metastatic spread in a mouse model. We therefore made use of an orthotopic A549-luc mouse model that was described earlier [13]. The authors showed that transpleural injection of A549-luc cells formed primary lung tumors that subsequently lead to mostly local metastatic lesions, which could be detected by BLI. We hypothesized that the enhanced migration and invasion capacity observed in vitro would translate in stronger metastatic spread in TGF-β-treated A549-luc cells when compared with untreated counterparts. Our pilot experiment showed that metastatic spread could be studied in this model. However, no differences in primary tumor growth and metastatic spread of epithelial and mesenchymal A549-luc cells was seen, mainly as a result of already extensive metastatic spread seen with the injected untreated A549-luc cells, which was much more extensive than previously reported [13]. Reduced numbers of injected A549 cells in combination with shorter tumor progression times will likely give a better window for studying differences in metastatic potential. Furthermore, we pre-treated the A549-luc cells with TGF-β for 3 days prior to injection, which may not lead to a persistent EMT phenotype of these cells in mice. In fact, in vitro we already noted that removal of TGF-β reversed the mesenchymal characteristics of A549 EMT cells indicating that continuous exposure to TGF-β is required to preserve the mesenchymal phenotype. Knock down of for example E-cadherin could be considered to induce a more permanent EMT in A549 cells, which was previously demonstrated to be an effective strategy in breast cancer cells [24].

Mordant et al also described the detection of CTCs in total blood obtained by venous blood puncture of these mice by using the CellSearch system allowing detection of EpCAM+, CD45-, cytokeratin positive tumor cells [13]. CTC counts ranged between 2 - 21 in 5 out of 6 animals. We anticipated that the higher invasive capacity of A549-luc EMT cells would translate in higher levels of CTCs. Moreover, detection of CTCs by BLI would allow detection that is irrespective of their epithelial or mesenchymal phenotype, which is a point of concern with the CellSearch system. However, our attempts to detect CTCs by means of BLI longitudinally in smaller blood samples obtained by cheek punctures or in total blood after venous tail puncture upon sacrificing the animals were unfortunately not successful. Under in vitro conditions we could detect around 50 A549-luc cells with the BLI system indicating that this method is likely not sensitive enough.
In other tumor types such as breast cancer a link between EMT and CSC has been established [7]. In particular, spheroid forming potential has been referred to as self-renewing capacity which is a feature of CSC [25]. Here, we found that TGF-β-induced EMT in A549 cells strongly enhanced spheroid-forming capacity when compared to untreated A549 cells that showed poor spheroid formation. TGF-β treatment thus appeared to enhance this in vitro CSC property in A549 cells. We also observed elevated expression of CSC markers such as OCT4 and SOX2 in TGF-β treated A549 cells. These findings are in agreement with a recent study showing enhanced CSC properties, such as elevated expression of OCT4, NANOG, SOX2 and CD133, increased spheroid formation and tumorigenic capacity in a mouse model of TGF-β treated LC31 lung cancer cells [26]. The same group also showed in the A549 model that TGF-β could induce EMT in both the CD133 positive CSC and CD133 negative non-CSC fractions that was associated with enhanced motility, however, not in the non-CSC (CD133-) fraction [27]. Overall, these and our findings are in support of EMT induction leading to enhanced CSC properties in A549 cells.

In conclusion, there is accumulating evidence for the involvement of EMT in drug resistance, invasion and CSC properties in NSCLC. This suggests that the targeting of EMT regulatory pathways may provide a promising novel approach for new therapy.

Conflict of Interest

There are no potential conflicts of interest.

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References


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Supplementary figures

**Supplementary figure 1.**
MTT assay depicting growth of A549 and A549+ TGF-β (72 hrs) cells, showing a growth delay in A549+ TGF-β cells after 2 and 3 days of TGF-β treatment.

**Supplementary figure 2.**
Bioluminescent imaging on different numbers of seeded A549-luc cells for generating a standard reference curve. A minimum of around 50 A549-luc cells suspended in RPMI+10% FCS media could be detected. Previously generated PC-3-luc [14] cells were included as a positive control and wells only containing RPMI+10% FCS as negative controls.