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Sustainability of CD24 expression, cell proliferation and migration, cisplatin-resistance, and caspase-3 expression during mesenchymal-epithelial transition induced by the removal of TGF-β1 in A549 lung cancer cells

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Abstract. Epithelial-mesenchymal transition (EMT) is a notable mechanism underlying cancer cell metastasis. Transforming growth factor β1 (TGF-β1) has been used to induce EMT; however, there is a lack of information regarding the role of TGF-β1 in mesenchymal-epithelial transition (MET). In the present study, EMT was induced in A549 lung cancer cells using TGF-β1 (TGF-β1-treated group) and MET was induced sequentially from the TGF-β1-treated group by removing the TGF-β1 (MET/return group). Untreated A549 lung cancer cells were used as a control. Characteristic features, including cancer stem cell markers [cluster of differentiation (CD)24, CD44 and CD133], cell proliferation and migration and diverse intracellular mechanisms, were observed in all groups. Using western blot analysis, the TGF-β1-treated group demonstrated increased vimentin and reduced E-cadherin expression, whereas the MET/return group demonstrated the opposite trend. Among cancer stem cell markers, the population of $CD24^{low}$ cells was reduced in the TGF-β1-treated group. Furthermore, the G2/M phase cell cycle population, cisplatin-sensitivity, and cell proliferation and migration ability were increased in the TGF-β1-treated group. These features were unaltered in the MET/return group when compared to the TGF-β1-treated group. Immunoblotting revealed an increase in the levels of SMAD3, phosphorylated SMAD3, phosphorylated extracellular signal-regulated kinase and caspase-3, and a decrease in active caspase-3 levels in the TGF-β1-treated group. Increased caspase-3 and reduced active caspase-3 levels were observed in the MET/return group, similar to those in the TGF-β1-treated group; however, levels of other signalling proteins were unchanged compared with the control group. EMT induced by TGF-β1 was not preserved; however, stemness-associated properties (CD24 expression, caspase-3 expression, cell proliferation and cisplatin-resistance) were sustained following removal of TGF-β1.

Introduction

Epithelial-mesenchymal transition (EMT) is a biological process observed in embryo neural crest formation (1). In order to migrate easily to distant locations, embryonic epithelial cells undergo EMT to become mesenchymal cells (2). In addition to embryonic cells, cancer cells also undergo EMT (3). This phenomenon was proposed as a cancer metastasis hypothesis, in which epithelial cancer cells downregulate E-cadherin to detach from the primary tumour (4). E-cadherin and vimentin, expressed in epithelial and mesenchymal cells, respectively, have been considered as key markers for EMT (5). Reports suggest that several factors, including Snail and Twist, are able to regulate E-cadherin expression (6,7). It was believed that epithelial-type cancer cells underwent EMT, changed to mesenchymal-type cancer cells, moved to a secondary organ and subsequently underwent mesenchymal-epithelial transition (MET) to form a secondary tumour mass (8). However, to the best of our knowledge, very little information has been reported regarding the MET process. A previous study

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Abbreviations: EMT, epithelial-mesenchymal transition; TGF-β1, transforming growth factor β1; MET, mesenchymal-epithelial transition; NSCLC, non-small cell lung cancer; STAT3, signal transducer and activator of transcription 3; NF-κB, nuclear factor-κB

Key words: transforming growth factor β1, epithelial-mesenchymal transition, mesenchymal-epithelial transition, caspase-3, cluster of differentiation 24, A549 lung cancer cell line

reported that paired box 2, bone morphogenetic protein 7 and Wilms tumour 1 were associated with MET during kidney formation in the embryo (9). The cancer stem cell model is a hypothesis associated with cancer metastasis. Cancer stem cells possess similarities with stem cells, sharing properties including self-renewal, differentiation and chemoresistance ability (10). With the aforementioned characteristics, it is believed that only cancer stem cells have an ability to develop a tumour mass in distant organs. There have been studies combining cancer stem cell and EMT theories in the analysis of mammary and lung cancer (11,12). Numerous markers for cancer stem cells have been identified in various types of solid and blood cancer. For example, cluster of differentiation (CD)133, CD44 and CD24 have been widely used to identify cancer types displaying stem cell properties (13). Lung cancer may be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (14). The incidence of NSCLC is 4 times higher, and it is more resistant to chemotherapy compared with small cell lung cancer (14). The prognosis of NSCLC patients is poor, and the 5-year survival rate was reported to be 5-15% (14). The A549 cancer cell line is an NSCLC cell line (15). A number of studies have been performed to correlate the association between EMT and cancer stem cells using the A549 cell line (15,16). However, to the best of our knowledge, little is known about MET, the reverse of the EMT process, and the association between MET and cancer stem cells following transforming growth factor β1 (TGF-β1)-induced EMT. Thus, the aim of the current study was to investigate the TGF-β1 microenvironment conditions affecting EMT and MET in the A549 lung cancer cell line. Additionally, properties associated with cancer stem cells were measured to reveal the association between EMT, MET and cancer stem cells.

Materials and methods

Cells, chemicals and reagents. A549 cell line was supplied by the Korean Cell Line Bank (Seoul, South Korea). Cisplatin, phosphate-buffered saline (PBS) and ribonuclease A were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). RPMI-1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). For flow cytometry, monoclonal antibodies for CD24 (cat. no. 130-095-953), CD44 (cat. no. 130-095-195) and CD133 (cat. no. 130-090-853) were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Protease inhibitor cocktail was obtained from Intron Biotechnology, Inc. (Seongnam, Korea). For western blotting, TGF-β1, radioimmunoprecipitation assay (RIPA) lysis buffer 3 and the primary polyclonal antibody against caspase-3 (cat. no. ADI-AAP-113) were supplied by Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Primary antibodies against E-cadherin (cat. no. BS1097) (polyclonal), SMAD3 (cat. no. AP0446) (polyclonal), and phosphorylated-SMAD3 (cat. no. BS64037) (polyclonal) were supplied by Bioworld Technology, Inc. (St. Louis Park, MN, USA). Primary antibodies against vimentin (cat. no. A301-620A) (polyclonal) and GAPDH (cat. no. A300-641A-M) (polyclonal) were provided by Bethyl Laboratories Inc. (Montgomery, TX, USA). Primary antibodies against signal transducer and activator of transcription 3 (STAT3; polyclonal), phosphorylated-STAT3 (monoclonal), extracellular signal-regulated kinase (ERK; polyclonal), phosphorylated-ERK (monoclonal), nuclear factor κB (NF-κB; polyclonal) and phosphorylated-NF-κB (polyclonal) were obtained from EMD Millipore (Billerica, MA, USA).

Cell culture. Cells were cultured in RPMI-1640 medium with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were maintained at 37˚C in an atmosphere of 5% CO_2 in a humidified incubator. The experiments were divided into 3 groups: Group 1 (control), TGF(-) received no treatment; group 2, TGF(+) treated with 10 ng/ml (17) TGF-β1 daily for 3 days; and group 3 (MET/return), TGF-β1-treated cells (group 2) were incubated in media for an additional 3 days following removal of TGF-β1.

Morphological analysis. The cells were grown in a coated cell culture dish (SPL Life Science, Pocheon, South Korea) and visualized without stain using an Olympus CKX41 optical microscope and Tomoro AcquCAM 3 (both Olympus Corporation, Tokyo, Japan) digital camera.

Flow cytometry. Immunostaining of A549 cells was performed as follows. Cells were blocked with 2% FBS reconstituted in PBS, and subsequently incubated with antibodies against CD24, CD44 and CD133 conjugated to allophycocyanin, fluorescein isothiocyanate and phycoerythrin (mouse anti-human, 1:11). The stained samples were analysed using a flow cytometer equipped with fluorescence-activated cell sorting BD CellQuest™ Pro software version 6.0 (BD Biosciences, Franklin Lakes, NJ, USA). The flow cytometric analysis was performed using isotype control antibodies and single colour stained samples in multivariate flow cytometry (18).

Cell cycle analysis. Cell cycle analysis was performed as described previously (19). A549 cells were seeded at a density of $1x10^5$ cells/well in 6-well plates. Following incubation, the cells were collected, washed with PBS, fixed with 70% ethanol and stored at 4˚C. To remove ethanol, stored cells were washed with PBS, ribonuclease A (50 μg/ml) was added and cells were incubated at room temperature for 5 min. Subsequently, the cells were stained with 10 μg/ml propidium iodide (PI), incubated at 37˚C for 10 min and counted using flow cytometry.

Cisplatin resistance test. TGF(-), TGF(+) and MET/return cells were incubated in 48-well cell culture plates in 400 μl medium. Cells were subsequently treated with cisplatin at a concentration of 100 ng/ml and incubated at 37˚C for 48 h. Subsequently, the cell viability was measured using WST-1 solution (EZ-Cytox kit; Daeillab Service Co., Ltd., Seoul, Korea) and the optical density at 450 nm was measured using an ELISA plate reader with Magellan™ Tracker software version 3.0.0.12 (Tecan Group Ltd., Männedorf, Switzerland) (19).

In vitro cell proliferation and migration assay. A549 cells were incubated in a gelatin-coated 6-well plate to form a 100% confluent monolayer prior to wounding. A wound was made (using a 1 ml pipette tip) by scraping across the monolayer, and the cells migrated in medium supplemented with 10% FBS (20).

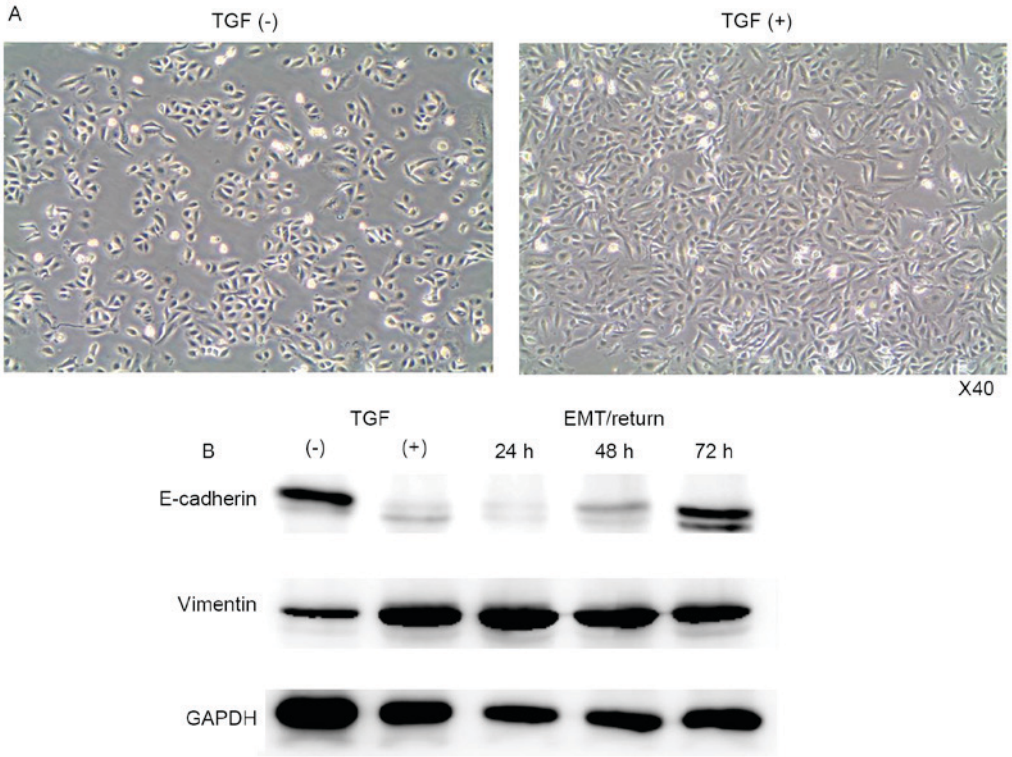

Figure 1. Morphological and physiological EMT and MET changes induced by TGF-β1. (A) Cell shape in RPMI cell culture media (10% fetal bovine serum). Magnification, x40. (B) Time-dependent immunoblotting in EMT and MET/return groups following removal of TGF-β1 from RPMI media. Every 24 h, A549 cells were collected and lysates were immunoblotted with antibodies against E-cadherin, vimentin and GAPDH (housekeeping gene). EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition; TGF, transforming growth factor.

Protein extraction and western blot analysis. Western blotting was performed as previously described (19). The whole-cell lysates were extracted with a mixture of RIPA lysis buffer 3, phosphatase inhibitor cocktail and protease inhibitor cocktail, according to the manufacturer's instructions. Lysate protein was centrifuged at 4˚C at 16,000 x g for 20 min. Lysate protein level was measured using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Aliquots of 10 μg cell lysate protein were resolved on 8% (*v/v*) SDS-PAGE gel, and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc.) for 90 min at 300 V. The protein-attached membrane was incubated with blocking buffer (TTBS, 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.05% Tween-20) supplemented with 5% (w/v) non-fat dry milk for 1 h at room temperature. The membrane was washed 5 times for 5 min each with TTBS and then incubated (overnight) with primary antibodies (rabbit anti-human; 1:1,000). Subsequently, the membrane was incubated (for 1 h) with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit; 1:5,000). Visualization of the protein bands was performed using enhanced chemiluminescence western blotting substrate (Chemiluminescent Sensitive Plus HRP Microwell and/or Membrane Substrate; SurModics, Inc., Eden Prairie, MN, USA).

Statistical analysis. The results are presented as the mean ± standard deviation. Comparison of the groups was performed using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS version 22 (IBM SPSS, Armonk, NY, USA).

Results

Induction of epithelial-mesenchymal transition. A549 cells were treated with TGF-β1 every 24 h for 3 days, at a concentration of 10 ng/ml. Morphological changes were observed daily by microscopy and images were recorded using a camera. After 72 h of treatment, the morphology of the A549 cells became fibroblast-like, with an elongated shape (Fig. 1A). To confirm EMT induction, western blotting was performed. E-cadherin (epithelial cell marker) was decreased, whereas vimentin (mesenchymal cell marker) was increased, following TGF-β1 treatment (Fig. 1B). In A549 cells, EMT was not fully induced until day 2 of treatment (data not shown).

Changes of mesenchymal-epithelial transition. Following induction of EMT, the TGF-β1 was washed from the cell culture media (group 3) to investigate the return of mesenchymal-type cells back to epithelial cells. Mesenchymal-type A549 cells were converted to epithelial-type cells after 3 days of incubation without TGF-β1 (Fig. 1B). The level of E-cadherin returned to normal, and the vimentin level was slightly reduced after 72 h.

Cancer stem cell marker properties. Cancer stem cell marker proteins, including CD24, CD44 and CD133 were measured using flow cytometry. In the control group, the population of $CD44^{high}$ was 96.07% (M2), $CD24^{low}$ was 37.76% (M1) and $CD133^{high}$ was 3.08% (M2) in A549 cells (Fig. 2). Following TGF-β1 treatment, $CD44^{high}$ and $CD133^{high}$ were slightly changed; however, the proportion of $CD24^{low}$ was reduced by

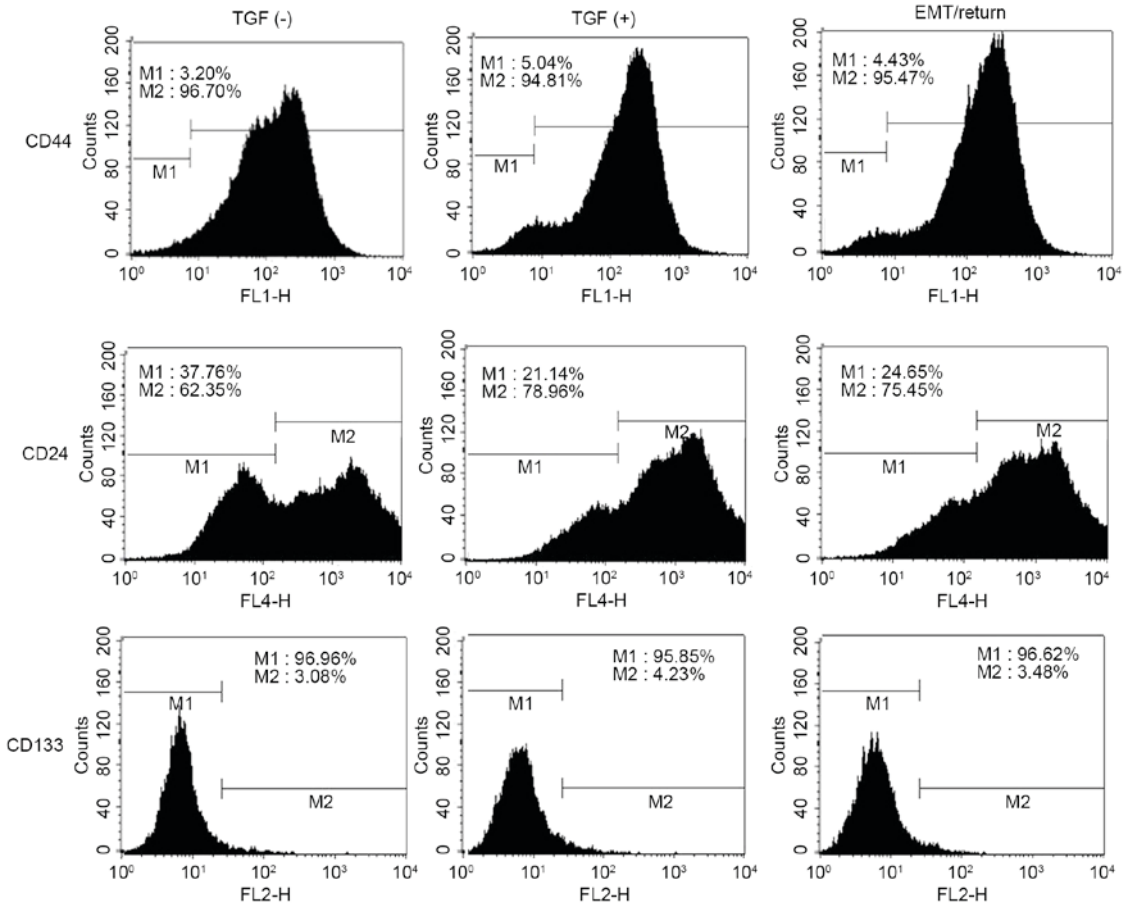

Figure 2. Population analysis of cancer stem cell marker-presenting cells. Flow cytometry of cancer stem cell markers: CD24, CD44 and CD133 in all treatment groups. CD, cluster of differentiation; TGF, transforming growth factor; EMT, epithelial-mesenchymal transition.

~15% (Fig. 2). Although the A549 cells in group 3 returned back to normal status, the profile of $CD24^{low}$ cells remained unaltered compared to group 2 (Fig. 2).

Cell cycle analysis, cisplatin resistance and wound healing assay. Untreated A549 cells demonstrated ~80% of the population in G0/G1 phase and 8.64% in G2/M phase. However, in the TGF-β1-treated group, the percentage of G2/M phase cells was almost doubled compared with the control group. In group 3, the percentage of cells in the G2/M phase was slightly reduced compared to group 2; however, it was still increased compared with the control (Fig. 3A). The A549 cells of the TGF-β1-treated and MET/return groups exhibited a significant sensitivity ($P<0.001$) to cisplatin treatment compared to the control (Fig. 3B). In the wound healing assay, TGF-β1-treated and MET/return groups exhibited increased proliferative and migration ability compared to the control (Fig. 3C).

Immunoblotting assay. In screening for the mechanism of action of TGF-β1 in A549 cells, 5 different signalling factors, including SMAD3, caspase-3, ERK, NF-κB and STAT3, were investigated. Levels of SMAD3 and the phosphorylated form of SMAD3 (known to be directly involved in TGF-β1 signalling) were increased in TGF-β1-treated cells, and returned to normal in the MET/return group (Fig. 4). By contrast, total caspase-3 was increased, and the active form of caspase-3 was decreased, in the TGF-β1-induced EMT and MET/return groups. NF-κB, STAT3 and ERK signalling were unchanged in all groups, with the exception of phosphorylated ERK. Phosphorylated ERK signalling was slightly increased in the TGF-β1-treated group, and returned to normal in the MET/return group (Fig. 4).

Discussion

The association between EMT and cancer stem cells was originally reported in 2008 by Mani *et al* (11). They sorted immortalized human mammary epithelial cells and observed that $CD44^{high}/CD24^{low}$ cells demonstrated vimentin expression and cancer stemness properties. However, in the present study, the portion of $CD24^{low}$ cells was decreased, $CD44^{high}$ was unchanged and the CD133+ lung cancer stem cell marker demonstrated negative results in TGF-β1-treated cells, findings which contrasted to other studies (11,21). However, the proportion of $CD24^{low}$ cells in the MET/return group was unchanged compared to that in the TGF-β1-treated group. From a hypothetical point of view regarding the association between cancer stem cells and EMT (3), this means that CD24 may be a key marker of cancer stemness in the A549 lung cancer cell line, a suggestion supported by Zheng *et al* (22). By contrast, it was reported that CD24 and CD44 were not considered as cancer stem cell markers in the A549 lung cancer cell line (23). In this previous study, ~70% of normal A549 cells were recorded as $CD44^{high}$ cells, and 30% were

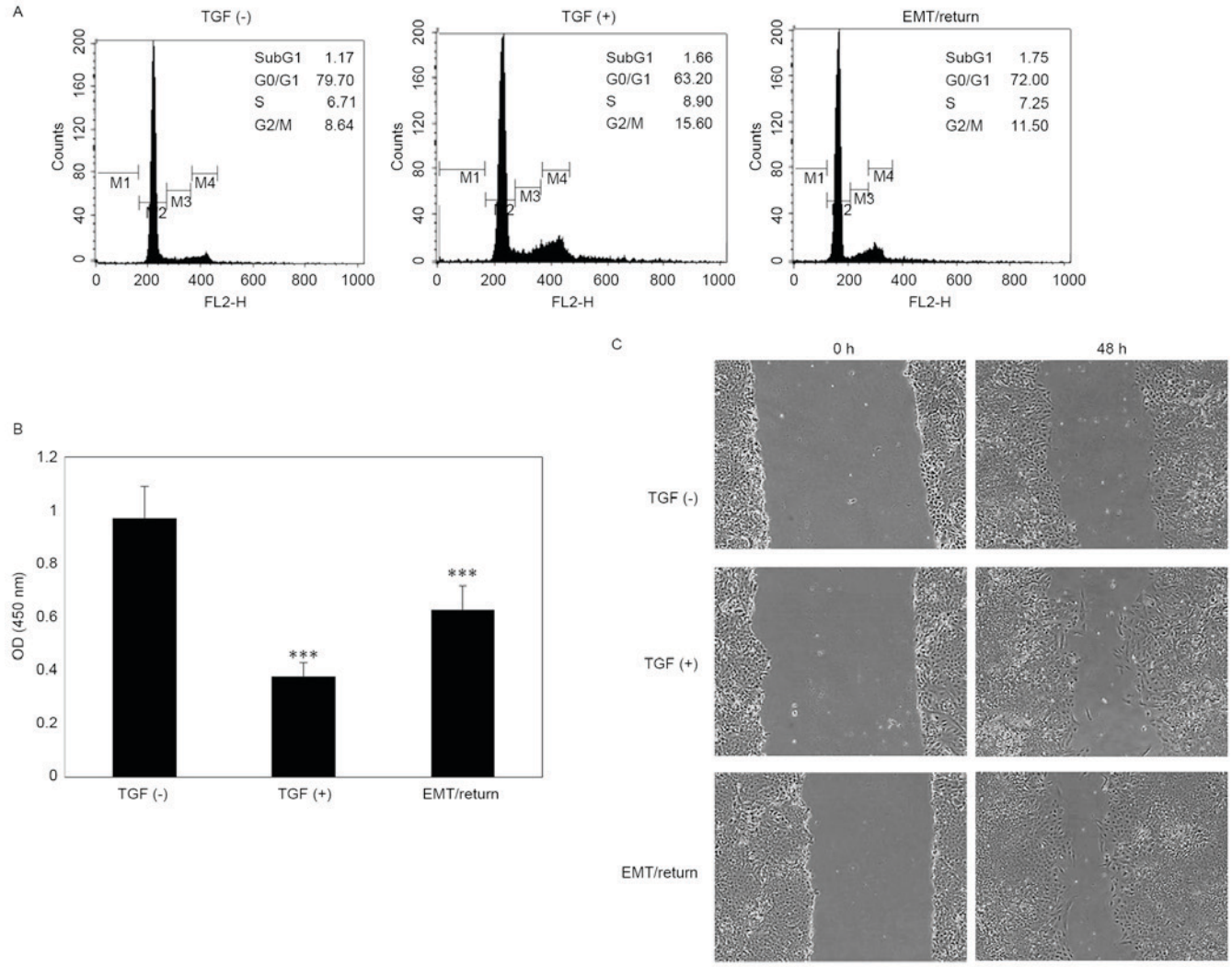

Figure 3. Cell cycle and cancer stemness-associated properties analysis. DNA staining of A549 cells using PI. (A) PI staining revealed single cell DNA content by cell cycle phase. (B) Cell viability assay following treatment with 100 ng/ml cisplatin for 24 h using a WST-1 assay kit. Data are expressed as the mean ± standard deviation (n=4). ***P<0.001 vs. TGF(-). (C) Cell proliferation and migration assay following cell incubation to 100% confluence, scratching with a pipette tip and visualization of the space using a digital camera. PI, propidium iodide; TGF, transforming growth factor; EMT, epithelial-mesenchymal transition; OD, optical density.

$CD24^{low}$ (23). In the present study, the percentage of $CD24^{low}$ cells was reduced in TGF-β1-treated and MET/return groups. This finding indicates that EMT induced by TGF-β1 is associated with CD24, and CD24 may be considered as a cancer stem cell marker. The results of the present study contrast with those of Roudi *et al* (23), who evaluated cancer stem cell markers without considering the EMT. Furthermore, the $CD24^{low}$ population was not significantly altered following the MET/return period, which means that TGF-β1 associated with cancer stem cells only during the commencement period.

Scheel and Weinberg (24) demonstrated that mesenchymal mammary cancer cells demonstrate resistance to chemicals and limited proliferation ability, findings which contrast with those of the present study. The present study reported that the TGF-β1-treated group exhibited sensitivity to cisplatin treatment, and high proliferation and migration ability compared to that in the control. The differences between studies may be attributed to different cell lines (mammary vs. lung cancer cell line), duration of treatment (24 h vs. 3 days) and the origin of the cells (primary vs. immortalized).

In the present study, the control A549 cells demonstrated higher cisplatin-resistance properties compared with the TGF-β1-treated group. It may be hypothesised that cancer stem-like cell properties, including self-renewal, chemoresistance and differentiation, should be considered independently when establishing a hypothesis connecting cancer stem cells and EMT. Wellner *et al* (12) proposed that zinc finger E-box binding homeobox 1 links EMT-activation and stemness, which was maintained via suppression of stemness-inhibiting microRNAs. However, this previous study focused only on tumorigenicity, by evaluating sphere culture, a feature known for its self-renewal ability (12). There may be a possibility that EMT could be partially connected with cancer stem cell properties, a suggestion supported by Xiao and He (25), who stated that neither reduced E-cadherin, nor induced N-cadherin, are associated with poor progression. In the present study, the MET/return group demonstrated little change regarding cell cycle, cisplatin-resistance, and proliferation and migration, compared with the TGF-β1-treated group. It may be suggested that EMT induced by TGF-β1 could be merely a trigger for

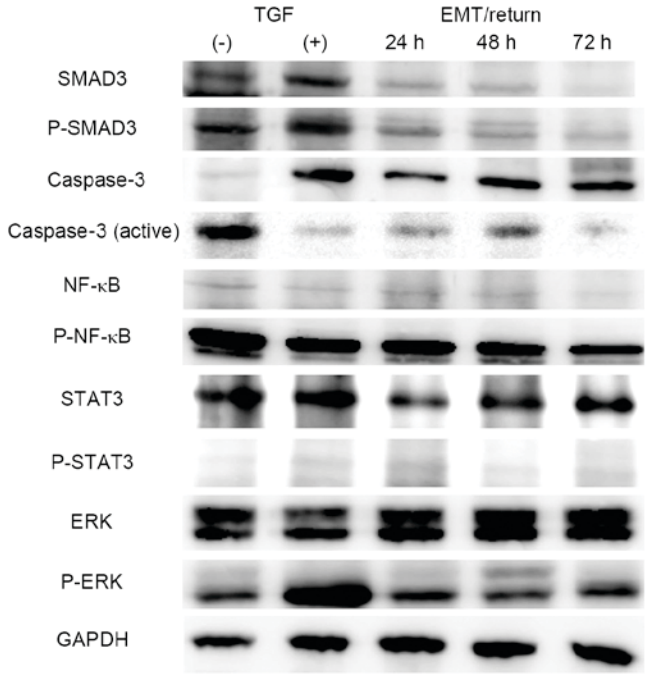

Figure 4. Mechanism screening by western blotting. Whole-cell lysates were immunoblotted with STAT3, ERK, NF-κB, caspase-3, SMAD3 and GAPDH (housekeeping gene) antibodies. STAT3, signalling transducer and activator of transcription 3; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; TGF, transforming growth factor; EMT, epithelial-mesenchymal transition; P, phosphorylated.

cancer stemness properties, with no further sustained effects following initiation.

TGF-β1 was reported to be involved in various cellular physiological changes, including proliferation, differentiation, apoptosis and EMT (26). TGF-β1 directly activated SMAD2 and SMAD3 via the TGF-β1 receptor (27). In the current study, SMAD3 and SMAD3 phosphorylation were activated in the TGF-β1-treated group; however, SMAD3 signalling disappeared within 24 h following TGF-β1 removal. This meant that TGF-β1 was acting as an EMT inducer in the TGF-β1-treated group, but not in the EMT/return group. It was reported that STAT3 was activated by TGF-β1 (28); however, this was not the case in the A549 cell line in the present study. By contrast, ERK was considered to be involved in TGF-β1-associated signalling and it was reported that ERK was activated by TGF-β1 in normal murine mammary gland epithelial cells (29). In the present study, phosphorylation of ERK was slightly increased in the TGF-β1-treated group; however, there was no response following TGF-β1 removal. NF-κB is a multi-transcription factor, which increases TGF-β1 transcription in rat mesangial cells (30). In the present study, level of NF-κB was measured to investigate the possibility of autocrine signalling of TGF-β1. Notably, there was no alteration in the level of NF-κB and phosphorylated NF-κB in A549 cells. It is difficult to rationalise the inconsistency between studies; however, the types of cell line, media and extra- and intracellular conditions are among the variables that may contribute to these differences. Concerning the apoptosis mechanism, it was reported that treatment with TGF-β1 may increase survivin, which promotes cell cycle progression and inhibits apoptosis during EMT (31). The results of the present study demonstrated that the active form of caspase-3 was decreased and G2/M phase cells were increased in the TGF-β1-treated and MET/return groups. It may be assumed that TGF-β1 is able to act as an initiator for an anti-apoptotic mechanism; however, additional research is strongly encouraged to support this assumption.

In conclusion, the present study revealed that mesenchymal cells of the A549 lung cancer cell line, induced by TGF-β1, return to epithelial cells in the absence of TGF-β1; however, the levels of CD24, caspase-3, cell proliferation and migration, and cisplatin sensitivity were unchanged during MET. It may be suggested that TGF-β1 acts as an initiator, but not a retainer, of properties associated with cancer stemness; however, additional research is required to confirm this hypothesis.

Acknowledgements

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