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Down-regulation of Par-3 expression and disruption of Par complex integrity by TGF- β during the process of epithelial to mesenchymal transition in rat proximal epithelial cells

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Key words: Par-3, Par complex, cell junction, epithelial to mesenchymal transition, TGF- β 1 □ proximal epithelial cells

Abstract

Epithelial-mesenchymal transition (EMT) is a fundamental mechanism of organ fibrosis and the initial step is disruption of cell junction and cell polarity. TGF- β has

been demonstrated as the most important mediator of EMT which is sufficient to initiate and complete the whole course of EMT, however, the detailed mechanism of TGF- β in modulating the disruption of cell junction still remains unclear. Par-3 is a component of Par complex which plays a crucial role in the establishment and maintenance of epithelial polarity. In this study, we found that TGF- β treatment resulted in a dose and time depend downregulation of Par-3 protein together with the suppression of E-cadherin expression and induction of α -SMA. The decreased Par-3 subsequently resulted in the redistribution of Par-6-aPKC complex from cell membrane to cytoplasm. Forced expression of exogenous Par-3 into rat proximal epithelial cells (NRK52E) led to a drastic blockage of TGF- β 1-induced E-cadherin suppression and α -SMA induction. In contrast, knockdown Par-3 expression by siRNA significantly enhanced TGF- β 1-induced E-cadherin suppression and α -SMA induction. These data indicate that downregulation of Par-3 and subsequent disruption of Par complex integrity might be one mechanism that TGF- β destroys cell polarity during EMT.

Introduction

In mature tubular epithelial cells, apical-basal polarity is maintained through the formation of several intercellular adhesion systems consisting of adhesion junctions, desmosomes, and tight junctions. The tight junction segregates the plasma membrane into structurally and functionally distinct basal and apical domains and the distribution of protein and lipid molecules in epithelial cells is asymmetric in these

two domains, which is required for proper polarization of epithelial cells [1-3]. However, these extremely differentiated epithelial cell phenotypes are not unchangeable. Under certain stimulations, differentiated epithelial cells can switch on a mesenchymal genetic program and form mesenchymal-like cells through a process called epithelial to mesenchymal transition (EMT)[4].

EMT is characterized by the disruption of epithelial junctions, loss of cell polarity, remodeling of actin cytoskeleton, alteration of cell-matrix adhesion, and increase in cell motility. EMT is a complex process, resulting from the interplay between numerous growth factors, cytokines, hormones, and extracellular cues. Among them, TGF- β is a sole factor which is sufficient to initiate and complete the whole course of EMT. TGF- β signals through type I (T β RI) and type II (T β RII) transmembrane receptor. Constitutive active T β RII activates T β RI, which in turn phosphorylates Smad2/3. Smad2/3 then bind to Smad4 and translocated into nucleus, where they regulate target gene transcription[5, 6]. It has been postulated that EMT at the cellular level occurs in a stepwise fashion. The earliest event of EMT is the disruption of cell junction, which manifests as the suppression of E-cadherin expression[7]. However, the exact molecular mechanism that TGF- β results in the disruption of cell-cell junctions remains unclear.

Recently, a signaling complex comprised of three proteins, partitioning-defective 3 (Par-3), Par-6 and atypical PKC (aPKC), emerged as a central paradigm in the establishment of apical junctions during adhesion-mediated cell polarization in various organisms ranging from worms to mammals [8-10]. The three proteins interact with each other to form a ternary complex and thus mutually regulate their functionality and localization[11]. The Par-3-Par-6-aPKC ternary complex (Par complex) was first identified in *Caenorhabditis elegans* as indispensable for the establishment of anterior/posterior polarity at the one-cell embryonic stage [12]. In *Drosophila* neuroblasts and epithelia, this protein complex localizes to the subapical regions, and plays a critical role in the establishment of apical-basal membrane polarity [13-15]. In mammalian epithelial cells, the complex localizes to the tight junction [16-18]. Studies using cultured epithelial cells demonstrated that the Par complex promotes the formation of epithelial tight junctions and thereby contributes to the establishment and maintenance of apical-basal polarity [17-19]. Considering the critical role of the Par complex in epithelial cell polarity, we hypothesize that TGF- β

regulates the expression and/or the integrity of this complex during the process of EMT. To test this hypothesis, we have investigated the effect of TGF- β 1 on the expression of Par-3, a key component of Par complex and the integrity of Par complex. Additionally, we examined the effect of over-expression of Par-3 on TGF- β 1-induced EMT in rat proximal tubular epithelial cells.

Materials and Methods

Reagents:

Normal rat kidney epithelial (NRK52E) cells were purchased from American Type Culture Collection (Manassas, VA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin/EDTA solution were purchased from Invitrogen (Carlsbad, CA); recombinant human TGF- β 1 (R&D Systems); TRIzol and reverse transcriptase were from Invitrogen (Carlsbad, CA, USA); Monoclonal mouse anti-HA antibody from Roche (Indianapolis, IN), antibody against E-Cadherin was from BD Biosciences (San Jose, CA), anti α -SMA (smooth muscle actin) was from DakoCytomation (Denmark), polyclonal anti-Par-3 antibody was from Upstate Biotechnologies (Charlottesville, VA), and polyclonal anti-Par-6 antibody was purchased from Santa Cruz Biotechnology Inc.(Santa Cruz, CA). pKH3-HA-Par-3b was a gift from Dr. Ian G. Macara (University of Virginia, Charlottesville, VA).

Cell culture and transfection

NRK52E cells were grown in DMEM medium supplemented with 10% FBS at 37°C in a humidified 5% CO₂ incubator. When 60% confluent, cells were cultured in serum free medium for 24 h before TGF- β 1 treatment.

For transient transfection, cells were transfected with 1 μ g plasmid DNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. To detect the expression of Par-3, cellular lysates were harvested 48 h after transfection. To examine the effect of overexpressed Par-3 on TGF- β 1-induced EMT, NRK52E cells were transfected with 1 μ g of pKH3-HA-Par-3 and then cultured in serum free medium for 24 hrs before TGF- β 1 treatment. Cellular lysates were harvested 72 hrs after TGF- β 1 treatment. To knockdown Par-3 expression, 4 μ g/ml of siRNA was transfected. The sequence of Par-3 siRNA is: 5-GGC AUG GAG ACC

UUG GAA GTT-3.

Stable transfectants were generated from NRK52E cells after selection with G418 800µg/ml. Ectopic expression of Par-3 in the stable cell line was confirmed by Western blot.

Real time PCR

Real-time PCR was performed with the ABI PRISIM 7000 sequence detection system. SYBR green real-time PCR mix (Invitrogen, Carlsbad, CA) was used for PCR reaction, with a primer concentration of 400nM. Primers for Real-time PCR were as follow:

Par-3: sense 5'- GAAGTCCCAAACCTGCGACAG -3'

antisense 5'- CTCCAGCCACCGGTCTACCATT -3'

GAPDH: sense 5'- CCCGCAGCCTCGTCTCATAGA -3'

antisense 5'- CTTCGGCCACCCTATCCAC -3'.

Reaction conditions were as follow: 95°C 2min for UDG incubation and 95°C 10min for UDG inactivation and DNA polymerase activation , then 40 repeats of 95°C for 15s, 62 °C for 1 min and each sample was performed triply in 25µl reaction volume. GAPDH was used as reference gene. Relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method based on Ct values for both target and reference genes in order to calculate the fold increase, results of real time PCR analysis are given as means \pm SD.

Immunoprecipitation and Western blot analysis

NRK52E cells were washed with PBS and scraped into lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM NaF, 10 mM Na₄P₂O₇, 1mM Na₃VO₅, 1 mM phenyl-methylsulfonyl fluoride, 10 µg/ml leupeptin and 20 µg/ml aprotinin). Protein was quantified by the Bradford assay (BioRad, Hercules, CA). To immunopreicipate endogenous complex of Par-3 and Par-6, polyclonal anti-Par-6 antibody was incubated with cell lysates at 4°C, followed by incubation with Protein-A-sepharose beads for 90 min at 4°C. Immunoprecipates were washed three times with lysis buffer and then subjected to 10% SDS-PAGE, and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). After blocking in 5% skim milk for 1 h at room

temperature, the membranes were incubated with indicated primary antibody at 4°C overnight followed by horseradish peroxidase-conjugated second antibody for 1 h at room temperature and detection by chemiluminescence (Amersham Life Science).

To prepare NP-40 insoluble fractions, the cells were lysed in 700 µl of ice-cold NP-40-IP buffer (25 mM Hepes/NaOH, pH7.4, 150 mM NaCl, 1% NP-40, 4 mM EDTA, 50 mM NaF, 1mM Na₃VO₅, 1 mM PMSF, 10 µg/ml leupeptin and 2 µg/ml aprotinin) by 30 min rotation at 4°C. After centrifugation the pellet was redissolved in 87.5 µl of 2 × SDS-sample buffer, and then subjected to western blot with anti-Par-6 antibody.

Immunofluorescence staining

NRK52E cells grown on glass coverslips were fixed in 2% paraformaldehyde for 15min at 4°C. After washing, cells were permeabilized in PBS containing 0.1% Triton-X100 for 5 min and then blocked with PBS containing 10% BSA for 30 min at room temperature. Cells were incubated for overnight at 4°C with indicated primary antibodies diluted in PBS (anti-Par-3 1:200, anti-Ecadherin 1:500, anti-α-SMA 1:500) containing 0.1% bovine serum albumin followed by detection with either FITC-conjugated goat anti-rabbit, FITC-conjugated goat anti-mouse or Cy3-conjugated donkey anti-mouse antibody in 5% BSA in PBS for 2 h at room temperature. Samples were mounted in fixation medium (Biomed, Foster City, CA). Images were analyzed and collected with Zeiss LSM 510 Confocal Imaging System (Zeiss, Germany).

Statistics

The results were expressed as mean ±SD from at least three experiments. Statistical analysis was carried out using SPSS11.0. Data were analyzed using one-way ANOVA followed by post-hoc test. A $p < 0.05$ was considered to be significant.

Results

TGF-β1 induces EMT in NRK52E cells and down-regulates Par-3 expression.

First of all, we assayed, by Western blot, for the amount of Par-3 protein in the lysate of NRK52E cells which were collected at various time points after TGF-β1 treatment. As seen in Fig. 1, TGF-β1 treatment led to a gradual decrease with time in the level of E-cadherin over 72 hr. In contrast, the level of α-SMA increased

drastically with TGF- β 1 treatment for 24 hr and reached a plateau up to 72 hr. Meanwhile, the amount of Par-3 started to decrease at 48 hr after TGF- β 1 treatment and plummeted to a level approximately 50% of that for the untreated cells at 72 hr (Fig. 1).

To further investigate whether TGF- β 1-induced Par-3 downregulation in NRK52E cells was dose-dependent, we treated the cells with incremental concentrations of TGF- β 1 from 2.5 to 20 ng/ml and assayed for Par-3 protein levels at 72 hr after TGF- β 1 treatment. The level of Par-3 decreased progressively with increased concentrations of TGF- β 1. At 20 ng/ml of TGF- β 1, Par-3 was decreased to approximately 30% of that in the control (untreated) cells (Fig. 2).

TGF- β 1 could regulate Par-3 level either at the mRNA level or post-translationally. To determine this, we next examined the Par-3 transcript during TGF- β 1-induced tubular EMT by real time PCR. As shown in Figure 3, Par-3 mRNA was gradually decreased after TGF- β 1 treatment in NRK52E cells, 10ng/ml TGF- β 1 treatment for 72hr resulted in a significant decrease of Par-3 transcript compared with that from cells treated with TGF- β 1 for 24hr.

We further confirmed the effect of TGF- β 1 treatment on the cellular morphology and expression of Par-3 in NRK52E cells by the immunofluorescence technique. Incubation of the cells with 10 ng/ml TGF- β 1 for 72 hr resulted in a significant loss of their epithelial phenotype and the cells displayed a fibroblast-like morphology identifiable by the presence of elongated lamellipodia (Fig.4A & B). To further characterize this cell morphological transition, we performed immunofluorescence staining of treated and untreated cells for examining various EMT markers. As shown in Fig. 4C and 4D, most TGF- β 1-treated cells lost the regular staining pattern and are characterized by a complete loss or fragmented staining of E-cadherin at the cell-cell junctions, whereas untreated cells exhibit a normal staining pattern for E-cadherin at the cell-cell junctions. To corroborate this observation, we stained the same cells for the mesenchymal marker α -SMA. Most untreated cells stained negative for α -SMA as expected. In contrast, approximately half of the TGF- β 1 treated cells were marked by strong α -SMA staining (Fig. 4E & F), and the pattern of staining was consistent with these cells acquiring a fibroblast-like phenotype. These data strongly suggest that NRK52E cells underwent EMT at prolonged TGF- β 1 treatment. To find out the

localization of Par-3 during EMT, we immunostained endogenous Par-3 in NRK52E cells before or after TGF- β 1 treatment (Fig. 4G & H). While Par-3 was detected prominently at the cell-cell junctions in normal NRK52E cells, it was significantly reduced in TGF- β 1 treated cells. Since many of the latter cells showed diffused cytosolic staining for Par-3, it is also likely that Par-3 relocates from the cell-cell junctions to cytosol during EMT. Taken together, these data indicate that TGF- β 1 downregulated Par-3 protein level in a time and dose-dependent manner in NRK52E cells.

TGF- β 1 treatment disrupts Par complex

Par-3 is a critical component of Par complex that targets Par complex to cell membrane[8, 9], we thus next examined the effect of the downregulation of Par-3 on other components of Par complex. By using western blot analysis, we found that the protein level of neither Par-6 nor aPKC was changed by TGF- β 1(10 ng/ml) treatment (Fig. 5A). We next examined the distribution of Par-6. As shown in Fig. 5B line 1 and 2, there is no significant change of total amount of Par-6 in NRK52E cells upon TGF- β 1 treatment, which is consistent with previous report[20], but TGF- β 1 treatment caused a remarkable decline of Par-6 in NP-40 insoluble fraction (Fig. 5B line 3 and 4) and an increase of Par-6 in NP-40 soluble fraction (Fig. 5B line 5 and 6). These results suggest that TGF- β 1 treatment caused a redistribution of Par-6 from cell membrane to cytosol.

We further performed immunoprecipitation to test the integrity of Par-3 with Par-6 and aPKC. The endogenous Par-6 was immunoprecipitated from either TGF- β 1 treated or untreated cells, and then immunoblotted with either anti-Par-3 or anti-aPKC antibody. As shown in Fig. 5C, the amount of Par-3 co-precipitated with Par-6 was remarkably reduced after TGF- β 1 treatment, but the amount of aPKC associated with Par-6 remained unchanged. This result suggests that TGF- β 1 treatment did not affect the association between Par-6 and aPKC.

Taken together, these data imply that the downregulation of Par-3 by TGF- β 1 treatment disrupted the Par complex on cellular membrane and redistributed Par-6-aPKC complex into the cytosol.

Overexpression of Par-3 blocks TGF- β 1-induced EMT

Since Par-3 expression was decreased by TGF- β 1 in EMT, we next investigated whether overexpression of Par-3 would affect the progress of EMT. HA-tagged Par-3 was transiently transfected into NRK52E cells. Overexpression of Par-3 was confirmed by Western blot using an anti-HA antibody (Fig. 6A). Expression of HA-Par-3 did not affect the protein level of E-cadherin or α -SMA in untreated NRK52E cells (Fig. 6A). Because E-cadherin is an epithelial marker that plays an essential role in the maintenance of cell junctions, and its loss is the earliest key cellular event during EMT, we next examined the effect of Par-3 overexpression on the cellular level of E-cadherin following TGF- β 1 treatment. As shown in Figure 6B, E-cadherin level was reduced after 10 ng/ml TGF- β 1 treatment for 72 hr in either untransfected cells (line “T”, Fig. 6B) or cells transfected with the empty vector (line “V”, Fig. 6B). Overexpression of Par-3 partially blocked the ability of TGF- β 1 to suppress E-cadherin expression (line “P”, Fig. 6B). In a parallel experiment, we found that overexpression of Par-3 significantly decreased TGF- β 1-induced α -SMA expression (Fig. 6B).

To further examine the effect of overexpression Par-3 on E-cadherin and α -SMA expression by immunofluorescence staining, we generated stably expressing Par-3 NRK52E cell line. Compared with control cells, Par-3 overexpressed cells were resistant to TGF- β 1 treatment and partially retained both E-cadherin and Par-3 staining at the cell-cell junction. Moreover, the number of cells positive for α -SMA staining was significantly reduced in Par-3 overexpressed cells (Figure 7).

Knockdown Par-3 expression promoted TGF- β 1-induced tubular EMT

To further confirm the role of Par-3 in EMT, we knocked down Par-3 expression by siRNA in NRK52E cells. As shown in Figure 8A, depletion of Par-3 did not affect the protein level of E-cadherin and α -SMA. TGF- β 1 treatment resulted in a decrease of E-cadherin expression and an induction of α -SMA expression. However, compared with non-targeting control siRNA transfected cells, E-cadherin expression was further reduced in Par-3 deficient cells after 10ng/ml of TGF- β 1 treatment for 72hr. Meanwhile, Par-3 knockdown dramatically enhanced TGF- β 1 induced α -SMA expression (Figure 8B). Collectively, these results indicate that Par-3 play a protective role in TGF- β 1-induced tubular EMT.

Discussion

In the present study, we have demonstrated that TGF- β 1 downregulated Par-3 protein expression in a dose- and time- dependent manner. The downregulation of Par-3 by TGF- β 1 caused a subsequent disruption of Par complex at the cell membrane and the redistribution of the dissociated Par-6-aPKC complex into cytoplasm. Overexpression of Par-3 could partially block TGF- β 1-induced tubular EMT.

Lines of in vitro and in vivo evidence indicate that TGF- β is a key initiator and promoter of EMT. Most studies suggest that TGF- β exerts its role mainly through regulating gene expression via Smad signaling pathway. Disruption of cell-cell contact is the first step of EMT and previous studies have shown that TGF- β suppresses gene expression of adhesion junction marker E-cadherin and tight junction protein ZO-1, claudin and occluding [21-23]. Lack of these transmembrane proteins would thus results in the disassembly of cell-cell adhesion. Consistent with previous studies, our study showed that TGF- β 1 downregulated Par-3 expression in a time and dose dependent manner at transcription level.

Cell junction consists of transmembrane proteins such as occluding, claudins and E-cadherin which are organized in intramembranous strands. These membrane proteins associate with a cytoplasmic plaque composed of a variety of peripheral membrane proteins including membrane-associated guanylate kinase (MAGUK) family of proteins ZO-1, -2, -3 or catenines that help to link membrane proteins to actin cytoskeleton. Recently, a couple of protein complexes such as Par complex, Scrib-Dlg-Lgl complex and Crumbs-Pals-Patj complex have emerged as important signaling proteins that regulate cell polarity in various organisms ranging from worms to mammals [8-10]. Despite the well recognized function of TGF- β on the regulation of transmembrane proteins, its role in these signaling complexes remains unclear. For the first time, we found that TGF- β 1 downregulated the protein level of Par-3 and further dissociated Par complex during the process of EMT, suggesting that disassembly of Par complex is one of the possible mechanisms that TGF- β 1 disrupts cell junction in EMT. Recently, Ozdamar et al have reported that TGF- β 1 regulated the phosphorylation of Par-6. Phosphorylated Par-6 then recruits the ubiquitin ligase Smurf1 to the receptor and leading to localized degradation of the RhoA GTPase which is required for the disruption of tight junction during EMT[20]. Since Par-3 interacts with Par-6, we speculate that the downregulation of Par-3 by TGF- β releases

Par-6 and thus allows it to be phosphorylated by TGF- β .

In epithelial cells, the Par complex plays an important role as a modulator of tight junction homeostasis and apical-basal polarity, with Par-3 as a key component of this complex. Suppression of Par-3 expression by RNA interference resulted in a dramatic disruption of TJ in mammalian epithelial cell [24]. Most recently, Gopalakrishnan et al reported that disruption of tight junction during ATP depletion involved the reduction of Par-3 phosphorylation and dysfunction of Par complex in MDCK cells [25]. These results are in accordance with our observation that Par-3 is down-regulated during the process of TGF- β 1-initiated tubular EMT when epithelial cells lose their polarity.

Taken together, disruption of cell junction by TGF- β 1 during EMT could involve the disassembly of signaling complex such as Par complex as well as the downregulation of transmembrane protein expression. A more detailed understanding of the effect of TGF- β 1 on these signaling complexes will help identify novel therapeutic targets and new therapeutic intervention approaches for EMT.

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Legend

Figure 1. TGF- β 1 suppressed Par-3 expression in NRK52E cells. (A) Western blot analysis was performed to examine the expression of Par-3, E-cadherin and α -SMA in NRK52E cells at treated with 10 ng/ml TGF- β 1 over 72 hr. A decrease of Par-3 expression was found at 48 hr and became more significant at 72 hr after TGF- β 1 treatment. GAPDH was used to verify equivalent loading. (B) Graphic presentation of relative abundance of Par-3, E-cadherin and α -SMA normalized to GAPDH. Data was given as mean \pm SD values of three independent experiments. * p <0.05 compared to untreated group.

Figure 2. TGF- β 1 suppresses Par-3 expression in a dose-dependent manner. (A) Western blot analysis was performed to examine the protein level of Par-3 in

NRK52E cells treated with various dose of TGF- β 1 for 72 hr. The decrease of Par-3 expression became significant at 10 ng/ml TGF- β 1 treatment. GAPDH was used to verify equivalent loading. (B) Graphic presentation of relative abundance of Par-3 normalized to GAPDH. Data was given as mean \pm SD values of three independent experiments. * p <0.05, ** p <0.01 compared to untreated group.

Figure 3. TGF- β 1 suppresses Par-3 expression at mRNA level. Bar graphs show averages of relative Par-3 mRNA abundance determined by quantitative RT-PCR in NRK52E cells treated with 10ng/ml TGF- β 1 at indicated time points. Data was given as mean \pm SD values of three independent experiments.

Figure 4. Expression and localization of Par-3, E-cadherin and α -SMA in NRK52E cells after TGF- β 1 treatment when assessed by immunofluorescence staining. Plots A & B: The epithelial morphology of these cells changed dramatically into fibroblast-like after TGF- β 1 treatment for 72 hr. Plots C & D: The expression of E-cadherin on the cellular membrane was decreased after 72 hr TGF- β 1 treatment. Plots E & F: α -SMA expression was significantly induced by TGF- β 1 treatment. Plots G & H: The staining of Par-3 at cellular membrane was decreased, but diffused cytosolic staining for Par3 was detected after TGF- β 1 treatment. Images were obtained by confocal microscopy.

Figure 5. (A) TGF- β 1 treatment did not change the protein level of Par-6 and aPKC but redistribute them into cytoplasm. The expression of GAPDH was analyzed as a loading control. NRK52E cells cultured with or without TGF- β 1 treatment for 72 hr were harvested by lysis buffer and then subjected to Western blot analysis with indicated antibodies. (B) TGF- β 1 treatment caused the redistribution of Par-6 to cytoplasm. NRK52E cells cultured with or without TGF- β 1 treatment for 72 hr were harvested by either lysis buffer or NP-40 IP buffer and then subjected to Western blot analysis with anti-Par-6 antibody. The expression of β -actin was analyzed in the same sample as a loading control. I, whole cell lysates from TGF- β 1 untreated cells; II, whole cell lysates from TGF- β 1 treated cells; III, NP-40 insoluble fraction from TGF- β 1 untreated cells; IV, NP-40 insoluble fraction from TGF- β 1 treated cells. V, NP-40

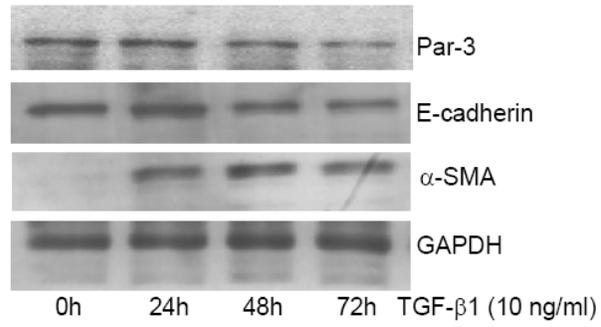
soluble fraction from TGF- β 1 untreated cells. VI, NP-40 soluble fraction from TGF- β 1 treated cells. (C) TGF- β 1 disassembled Par complex. NRK52E cells cultured with or without TGF- β 1 for 72 hr were harvested by lysis buffer and proteins separated by centrifugation. The supernatants were collected and 1 mg protein was subjected to immunoprecipitation with anti-Par-6 antibody and precipitated protein was analyzed by Western blot analysis using either anti-Par-6, anti-aPKC antibody or anti-Par-3 antibody. Data shown are representative of three independent experiments.

Figure 6. Overexpression of Par-3 inhibited TGF- β 1-induced EMT. (A) Overexpression of Par-3 did not affect E-cadherin and α -SMA expression in the absence of TGF- β 1. NRK52E cells were transiently transfected with either pKH3-HA-Par-3 or pKH3 empty vectors. 48 hr after transfection, whole cellular lysates were prepared, quantitated and equivalent amounts of protein resolved by SDS-PAGE and immunoblotted with indicated antibodies. Overexpression of Par-3 was detected by monoclonal anti-HA antibody. GAPDH was used to verify equivalent loading. (B) Overexpression of Par-3 blocked TGF- β 1-induced E-cadherin suppression and attenuated TGF- β 1-induced α -SMA expression. NRK52E cells were transiently transfected with either pKH3-HA-Par-3 or pKH3 empty vectors followed by 10 ng/ml of TGF- β 1 treatment for 72 hr, and then cellular lysates were harvested to detect E-cadherin, α -SMA and Par-3 expression by Western blot analysis. (C) untransfected cells; (T) untransfected cells treated with TGF- β 1; (V) pKH3 empty vector transfected cells treated with TGF- β 1; (P) pKH-HA-Par-3 transfected cells treated with TGF- β 1. E-cadherin/GAPDH ratio was given as mean \pm SD from three independent experiments. # p >0.05 compared to TGF- β 1 treated untransfected group, * p <0.05 compared to both TGF- β 1 treated untransfected group and TGF- β 1 treated empty vector transfected group.

Figure 7. Stable transfection of Par-3 into NRK52E cells partially blocked TGF- β 1-induced Par-3, E-cadherin suppression and α -SMA induction. Laser confocal microscopy images showing Par-3(A, B), E-cadherin(C□D) and α -SMA(E□F) immunofluorescence in both control NRK52E cells (A, C, E) and pKH3-HA-Par-3 (B, D, F) stably expressing NRK52E cells upon TGF- β 1 treatment for 72hr.

Figure 8. Knockdown Par-3 expression by siRNA promoted TGF- β 1-induced EMT. (A) NRK52E cells were transiently transfected with Par-3 and non-targeting siRNA as described in methods section. Cells were lysed and Western blot was performed to detect the expression of Par-3, E-cadherin and α -SMA. (B) NRK52E cells were transiently transfected with either Par-3 or non-targeting siRNA followed by TGF- β 1 treatment for 48hr, and then cells lysate were harvested to detect the expression of E-cadherin and α -SMA by Western blot analysis. E-cadherin/GAPDH ration and α -SMA /GAPDH ratio were given as mean \pm SD from three independent experiments. (C) untransfected cells; (T) untransfected cells treated with TGF- β 1; (N) non-targeting siRNA transfected cells treated with TGF- β 1; (P) Par-3 siRNA transfected cells treated with TGF- β 1. # $p>0.05$ compared to TGF- β 1 treated untransfected group, * $p<0.05$ compared to both TGF- β 1 treated untransfected group and TGF- β 1 treated non-targeting siRNA transfected group.

A



B

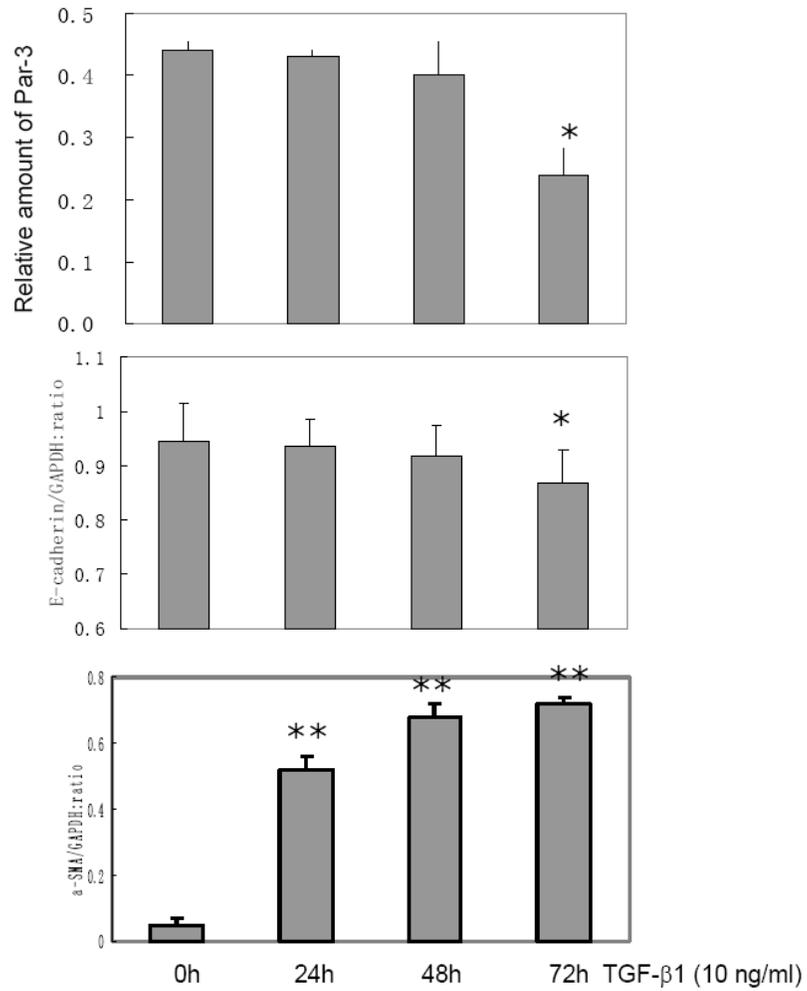
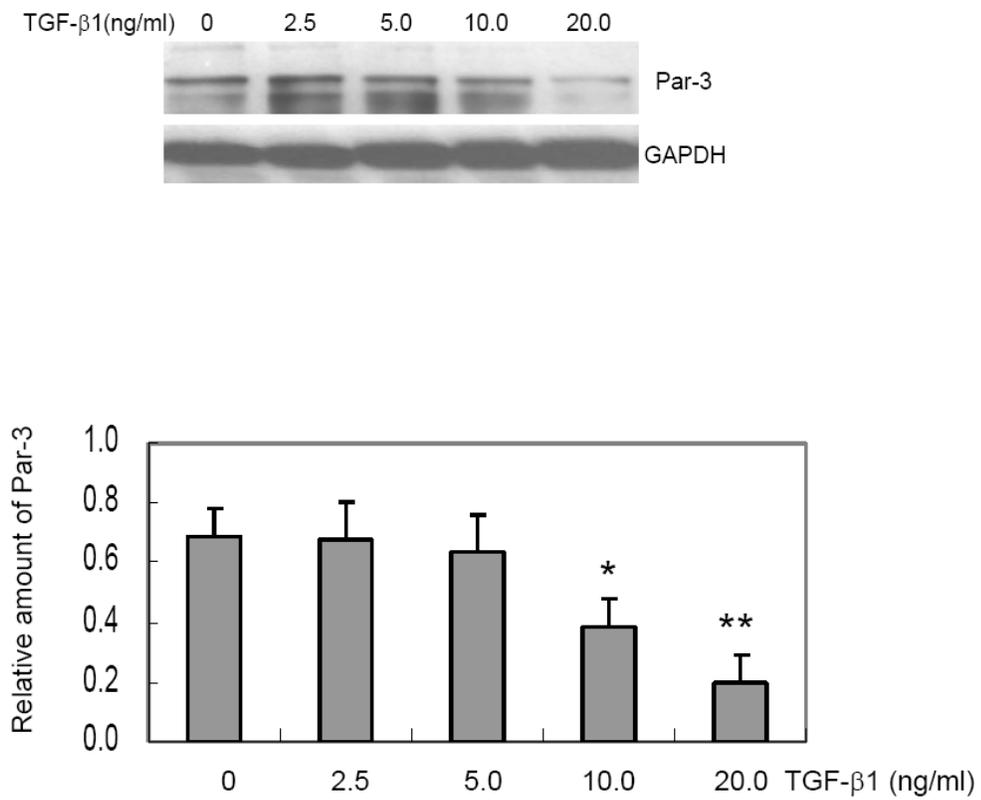
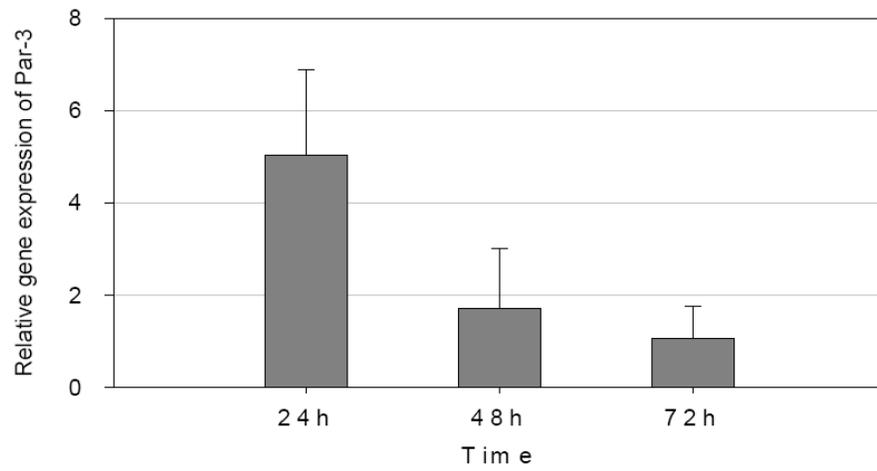


Figure 1

**Figure 2**

**Figure 3**

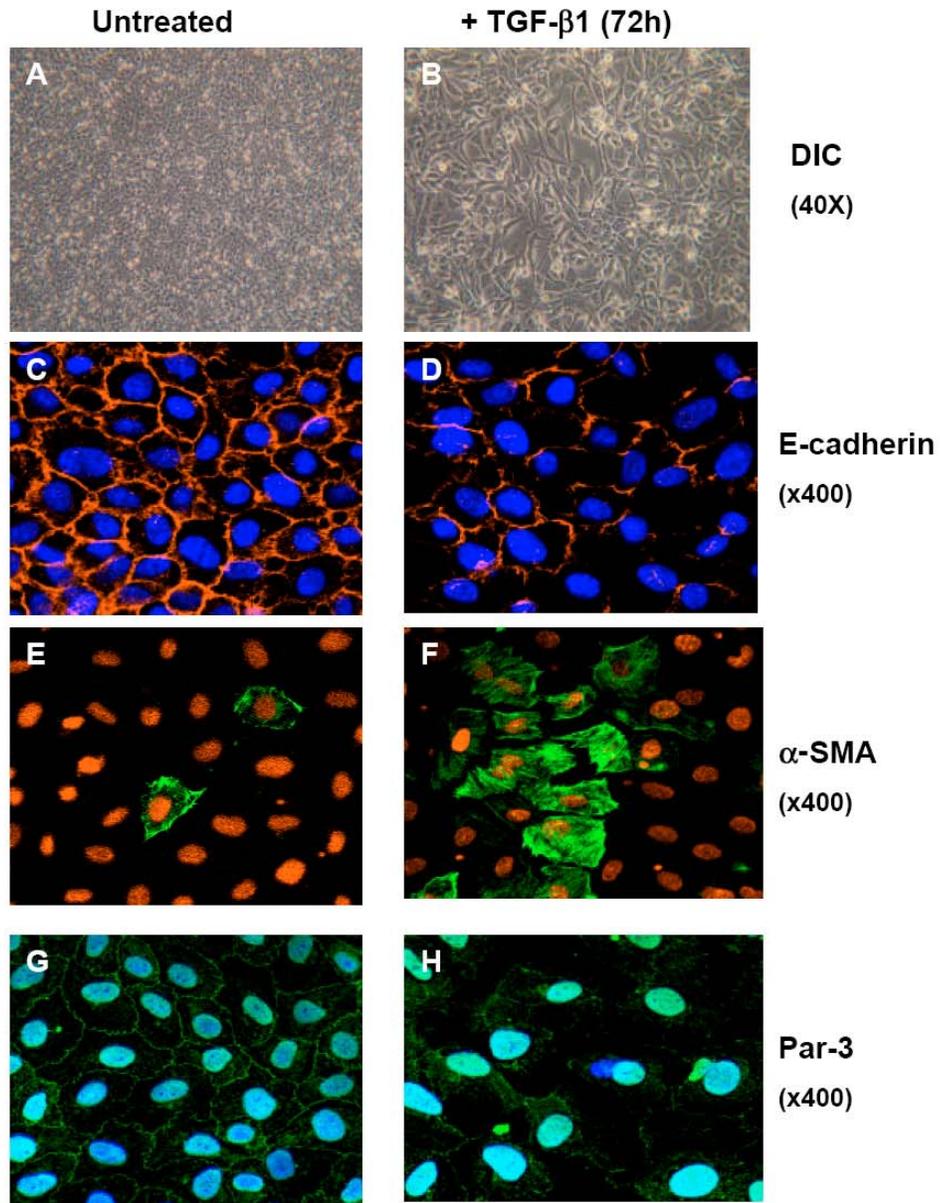


Figure 4

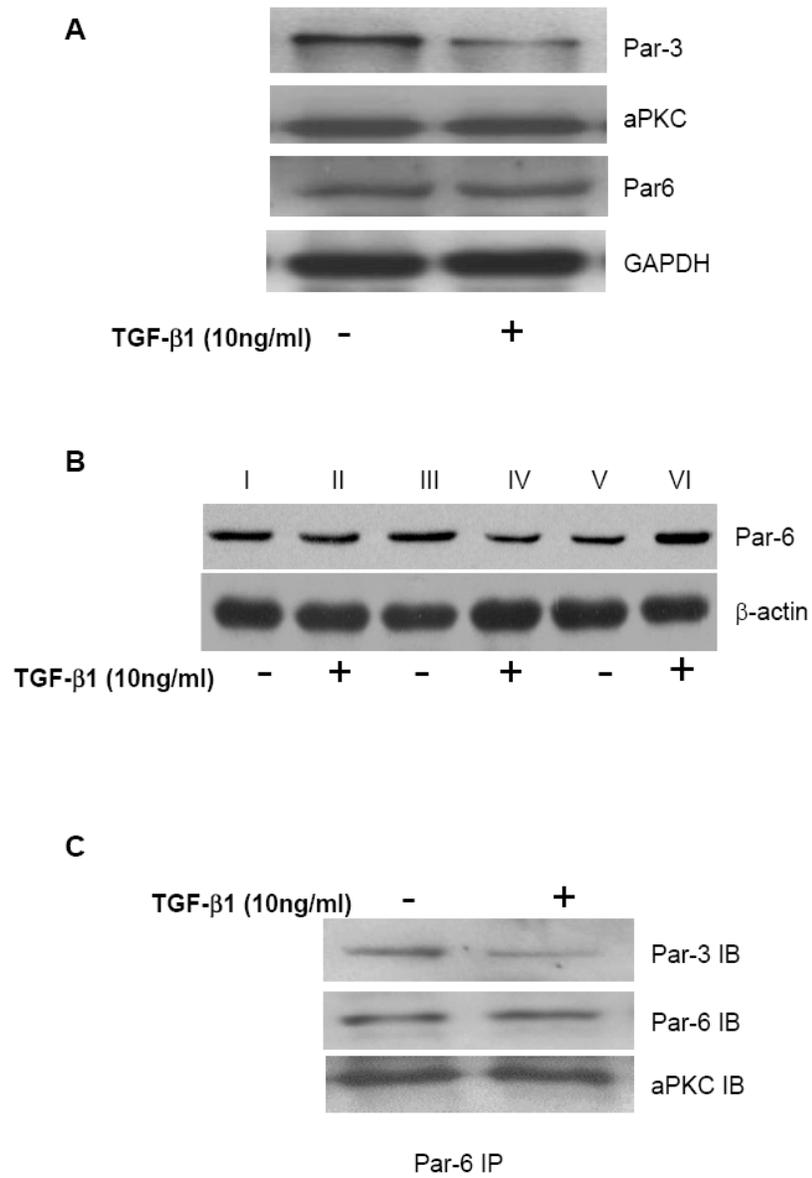


Figure 5

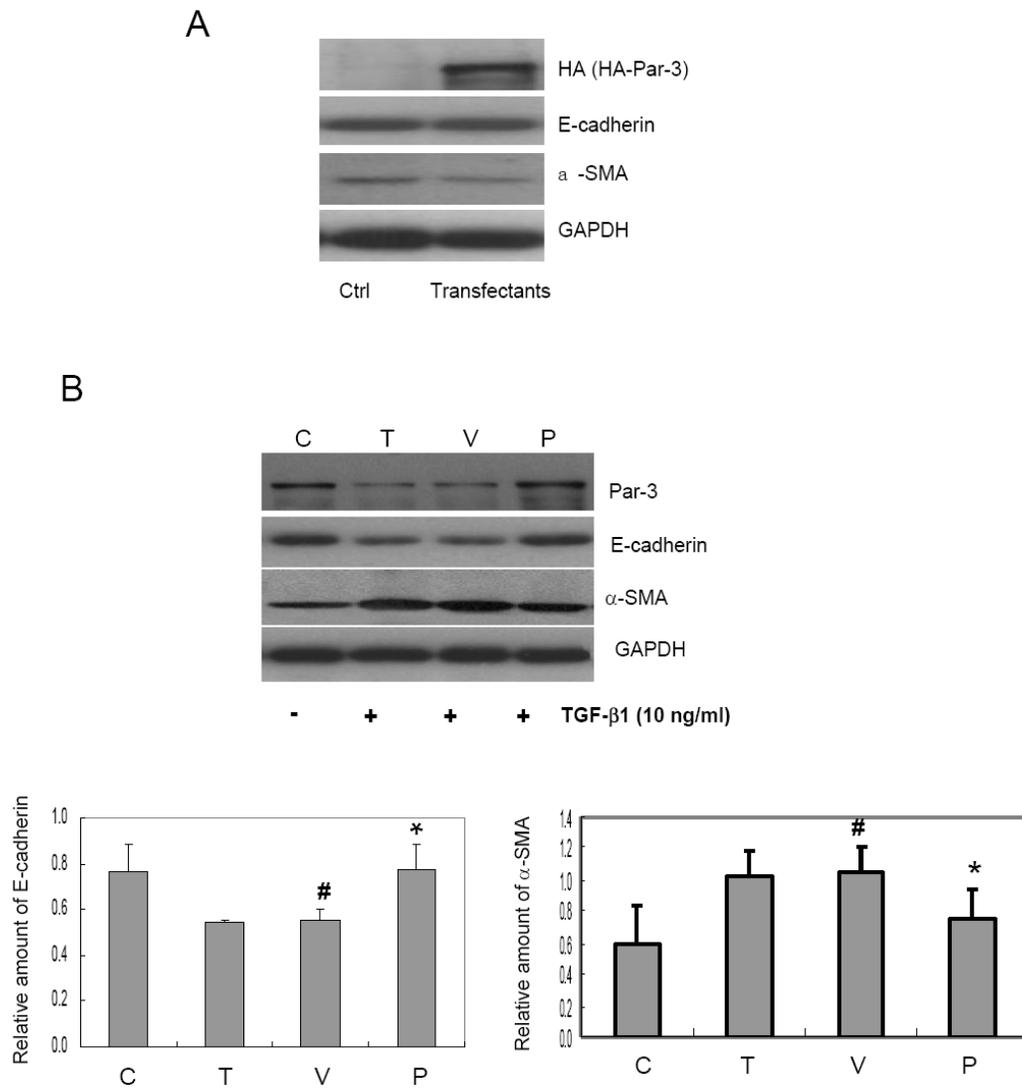


Figure 6

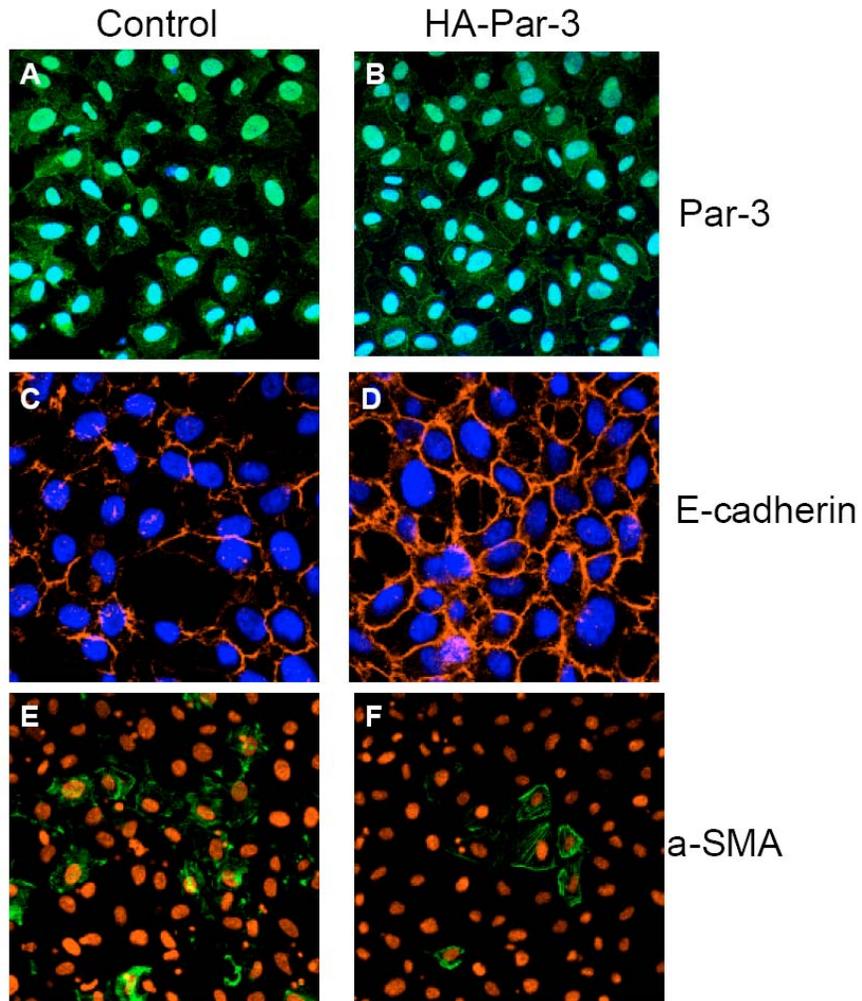


Figure 7

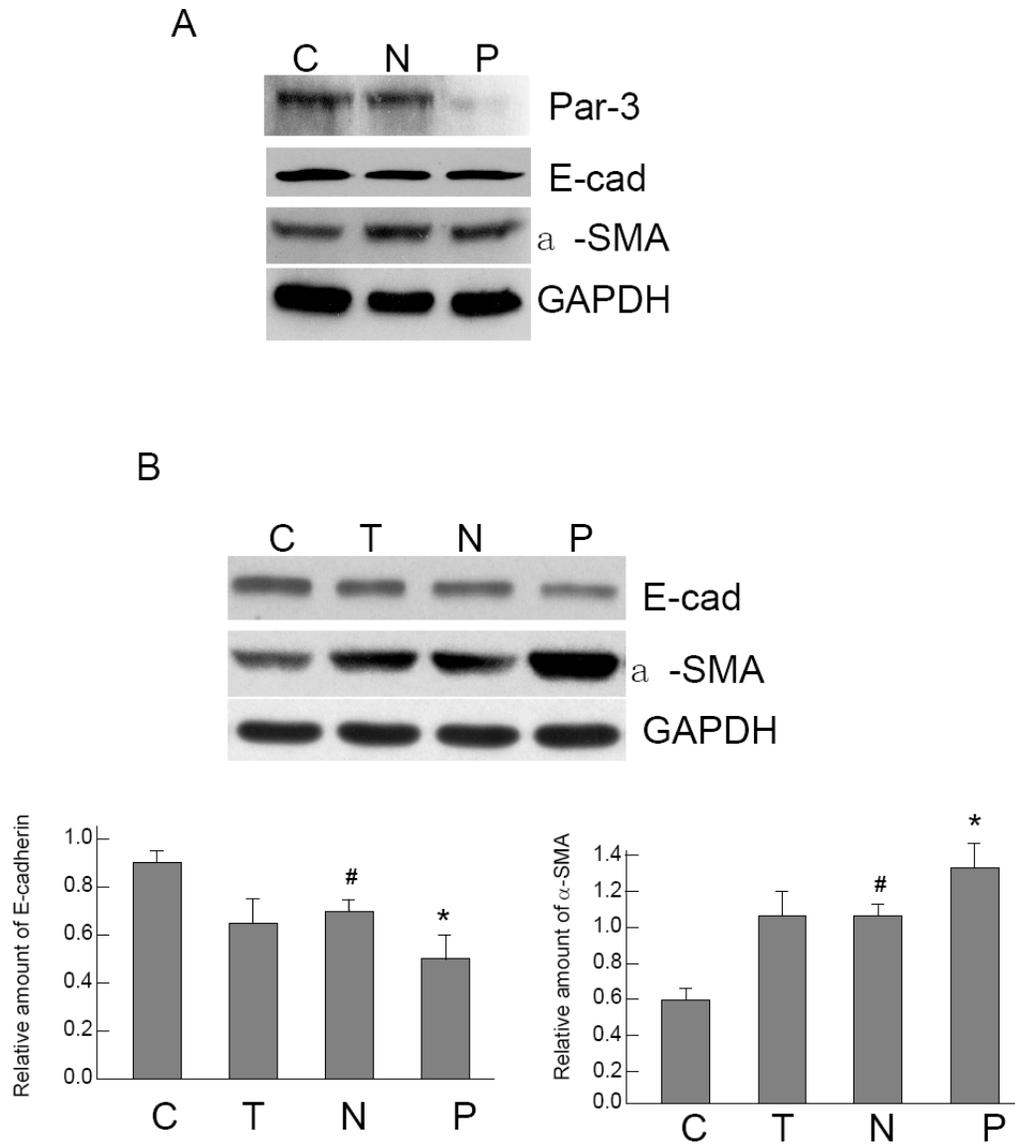


Figure 8