

# Epithelial–Mesenchymal Transition (EMT) Induced by TNF- $\alpha$ Requires AKT/GSK-3 $\beta$ -Mediated Stabilization of Snail in Colorectal Cancer

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## Abstract

Chronic inflammation-promoted metastasis has been considered as a major challenge in cancer therapy. Pro-inflammatory cytokine TNF $\alpha$  can induce cancer invasion and metastasis associated with epithelial–mesenchymal transition (EMT). However, the underlying mechanisms are not entirely clear. In this study, we showed that TNF $\alpha$  induces EMT in human HCT116 cells and thereby promotes colorectal cancer (CRC) invasion and metastasis. TNF $\alpha$ -induced EMT was characterized by acquiring mesenchymal spindle-like morphology and increasing the expression of N-cadherin and fibronectin with a concomitant decrease of E-cadherin and Zona occludin-1(ZO-1). TNF $\alpha$  treatment also increased the expression of transcription factor Snail, but not Slug, ZEB1 and Twist. Overexpression of Snail induced a switch from E-cadherin to N-cadherin expression in HCT116 cells, which is a characteristic of EMT. Conversely, knockdown of Snail significantly attenuated TNF $\alpha$ -induced EMT in HCT116 cells, suggesting that Snail plays a crucial role in TNF $\alpha$ -induced EMT. Interestingly, exposure to TNF $\alpha$  rapidly increased Snail protein expression and Snail nuclear localization but not mRNA level upregulation. Finally, we demonstrated that TNF $\alpha$  elevated Snail stability by activating AKT pathway and subsequently repressing GSK-3 $\beta$  activity and decreasing the association of Snail with GSK-3 $\beta$ . Knockdown of GSK-3 $\beta$  further verified our finding. Taken together, these results revealed that AKT/GSK-3 $\beta$ -mediated stabilization of Snail is required for TNF $\alpha$ -induced EMT in CRC cells. Our study provides a better understanding of inflammation-induced CRC metastasis.

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## Introduction

Chronic inflammation has been identified to be intimately associated with tumorigenesis [1,2]. Increasing evidences have proved that the inflammatory tumor microenvironment plays a crucial role in tumor development and metastasis [3]. Tumor microenvironment is largely orchestrated by inflammatory cells, which facilitate extracellular matrix breakdown, angiogenesis, and tissue remodeling, thus promote tumor cell motility [4]. Furthermore, tumor cells themselves can secrete proinflammatory cytokines which contribute directly to malignant progression [5]. The complex interactions between the tumor and inflammatory cells mediated by inflammatory cytokines are an essential aspect of the tumor microenvironment [6]. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), a proinflammatory cytokine predominantly produced by macrophages, is a key molecule regulating the inflammatory processes in tumor promotion. Mounting evidences suggested that TNF $\alpha$  mediates many critical processes of tumor progression, including oncogene activation, DNA damage, and tumor metastasis [7].

Epithelial–mesenchymal transition (EMT), an essential phenotypic conversion during embryonic development, tissue remodeling, and wound healing, plays an indispensable role in tumor

invasion and metastasis [8–10]. EMT is a reversible process that often occurs at the invasive front of many metastatic cancers [11]. EMT can be triggered by different signals received from tumor microenvironment, such as TGF $\beta$ , EGF, WNTs and Notch [12]. During the processes of EMT, epithelial cells loss intercellular adhesion, acquire fibroblast-like characteristics and increase migratory and invasive properties [13]. One of the most well-defined features of EMT is the loss of E-cadherin expression [8]. A group of transcription factors, including Snail, Slug, ZEB1, Twist, have been implicated in the control of EMT [14]. Snail, a zinc-finger transcription factor first identified in *Drosophila*, has been proved as a key EMT regulator [15]. Studies showed that Snail represses E-cadherin transcription by binding to the E-box site in the promoter of E-cadherin [16–18]. The roles of Snail in EMT regulation have been reported in many types of cancer such as breast carcinoma, ovarian carcinoma, etc. [14,18,19]. Silencing of Snail by stable RNA interference induces the complete mesenchymal to epithelial transition (MET) in MDCK-Snail cells, which associates with the inhibition of invasion [20]. In addition, high expression of Snail also correlates with tumor grade, recurrence, nodal metastasis and poor outcomes in patients [21–25]. Several

inflammatory mediators such as TGF $\beta$ , hypoxia and IL-6 can upregulate Snail and therefore trigger EMT [3]. These findings highlight the importance of the microenvironment in regulation of Snail and in the initiation of EMT.

The colorectal cancer (CRC) is a major worldwide health concern. Most deaths from CRC are due to metastases that are resistant to conventional therapies. EMT is a highly relevant issue to CRC metastasis [26]. However, the role of TNF $\alpha$  in EMT of CRC is rarely investigated and the underlying molecular mechanism remains unclear. Here we showed that TNF $\alpha$ -induced EMT by stabilizing Snail in HCT116 and Caco-2 cells. We also demonstrated that TNF $\alpha$  stabilizes Snail by activating AKT pathway and thereby inhibiting GSK-3 $\beta$  activity and decreasing the association of GSK-3 $\beta$  and Snail.

## Materials and Methods

### Chemicals and Reagents

NF- $\kappa$ B inhibitor BAY11-7082, ERK inhibitor PD98059, p38 MAPK inhibitor SB-203580, PI3K inhibitor LY294002, GSK-3 $\beta$  inhibitor lithium (LiCl) and proteasome inhibitor MG132 were obtained from Sigma-Aldrich (St Louis, MO). Primary antibodies against E-cadherin, Zona occludin-1(ZO-1), Snail, ZEB1, p-GSK-3 $\beta$  (ser9), GSK-3 $\beta$ , p-Akt (Ser473), Akt, and  $\beta$ -catenin were obtained from Cell Signaling Technology (MA, USA). Primary antibody against Histone H2A.X was obtained from Bioworld (Bioworld Technology, Minneapolis, MN, USA). Protein A/G Sepharose and primary antibodies against N-cadherin, ubiquitin,  $\beta$ -actin,  $\alpha$ -tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody to fibronectin was obtained from Boster Biological Engineering. Horseradish peroxidase (HRP)-conjugated secondary antibody, Alexa Fluor 488/594 conjugated secondary antibody, DAPI and lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant human TNF $\alpha$  protein was bought from PeproTech. PrimeScript<sup>®</sup> RT reagent Kit and SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> were products of TaKaRa. E.Z.N.A.<sup>®</sup> HP Total RNA Kit was bought from Omega Bio-Tek (Doraville, USA). Smart pool siRNA against human Snail and GSK-3 $\beta$  were from RIBOBIO.

### Cell Culture

The HCT116 and Caco-2 colorectal carcinoma cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HCT116 cells were maintained in McCoy's 5a culture medium (Gibco BRL) supplemented with 10% fetal bovine serum, and Caco-2 cells were cultured in DMEM culture medium (Gibco BRL) supplemented with 10% fetal bovine serum under a humidified 5% CO<sub>2</sub> atmosphere at 37°C in incubator.

### Transwell Migration and Invasion Assay

Migration and invasion assays were performed in Boyden chambers. The polycarbonate filters (8  $\mu$ m pore size, Corning) pre-coated with Matrigel Matrix (BD Biosciences) were used for invasion assay, and uncoated filters were used for migration assay. Cells ( $1 \times 10^5$ ) in 300  $\mu$ l medium (containing 0.1% FBS) with or without 20 ng/ml TNF $\alpha$  were seeded in the upper chamber. Then 600  $\mu$ l medium with 10% FBS was added to the lower chamber and served as a chemotactic agent. After 24 h incubation, for migration, the cells migrated and adhered onto the lower chamber were fixed in 4% paraformaldehyde for 20 min, stained with hematoxylin and counted under upright microscope (5 fields per chamber). For invasion, the cells in the upper chamber were fixed in 4% paraformaldehyde for 20 min.

Then the matrigel was mechanically removed from the filter with a cotton swab. The cells adhering to the under-side of the filter were stained with hematoxylin and counted under upright microscope (5 fields per chamber). Each migration and invasion assay was repeated in three independent experiments.

### Gene Over-expression and RNA Interference

The cells were seeded on a 6-well plate ( $2 \times 10^5$  cells/well) and left in culture until the next day. They were then transfected with 2  $\mu$ g plasmid vector or 100 pmol siRNA oligomer mixed with lipofectamine 2000 reagent in serum reduced medium according to the manufacturer's instructions. Medium was changed to complete culture medium 6 h later, and the cells were incubated at 37°C in a CO<sub>2</sub> incubator for another 24 to 48 h before harvest.

### Quantitative Real-Time PCR

Total mRNA of the cells was extracted after treatment for the indicated time. First strand cDNA synthesis was generated from 500 ng of total RNA. Quantification of target and reference (GAPDH) genes was performed in triplicate on LightCycler<sup>®</sup> 480 II (Roche, Applied Science). The primers used in each reaction were as follows: E-cadherin forward 5'-TACACTGCCAGGAGCCAGA-3' and reverse 5'-TGCCACCAGTGTCCGGATTA-3'; N-cadherin, forward 5'-CGAATGGATGAAAGACCCATCC-3' and reverse 5'-GGAGCCACTGCCCTTCATAGTCAA-3'; Snail, forward 5'-GACCACTATGCCGCGCTCTT-3' and reverse 5'-TCGCTGTAGTTAGGCTTCCGATT-3'; ZEB1, forward 5'-TACAGAACCCAACTTGAACGTCACA-3' and reverse 5'-GATTACACCCAGACTGCGTCACA-3'; Twist, forward 5'-GGAGTCCGCAGTCTTACGAG-3' and reverse 5'-TCTGGAGGACCTGGTAGAGG-3'; Slug, forward 5'-TTCGGACCCACACATTACCT-3' and reverse 5'-GCAGT-GAGGGCAAGAAAAAG-3'; GAPDH, forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-TGGTGAAGACGCCAGTGGA-3'. After normalized to GAPDH gene, expression levels for each target gene were calculated using the comparative threshold cycle (CT) method. The  $\Delta$ ct values were calculated according to the formula  $\Delta$ ct = ct (gene of interest) - ct (GAPDH) in correlation analysis, and the  $2^{-\Delta\Delta$ ct was calculated according to the formula  $\Delta\Delta$ ct =  $\Delta$ ct (control group) -  $\Delta$ ct (experimental group) for determination of relative. Data are presented as the mean  $\pm$  standard deviation (SD) from three independent experiments.

### Western Blotting Analysis

The cells were washed three times with ice-cold phosphate buffer solution (PBS) and then lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Na-deoxycholate, 5  $\mu$ g/ml aprotinin, 5  $\mu$ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Equal amounts of protein samples were loaded per well and separated on SDS-polyacrylamide gels, and then electrophoretically transferred onto PVDF membranes. Following blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with primary antibodies (1:1,000 dilution) at 4°C overnight and then incubated with HRP-conjugated secondary antibodies (1:5,000 dilution) for 2 h at room temperature. Specific immune complexes were detected using Western Blotting Plus Chemiluminescence Reagent (Life Science).

## Immunofluorescence

The cells were cultured on chamber slides, serum starved for 12 h, then exposed to TNF $\alpha$  for the indicated time. Cells were washed three times with PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 for 10 min. After blocking with goat serum for 2 h at room temperature, cells were incubated with antibodies against E-cadherin, N-cadherin, fibronectin, ZO-1 or Snail (1:100 dilution) at 4°C overnight. Slides were washed three times with PBS and incubated with Alexa Fluor 488 or Alexa Fluor 594-conjugated secondary antibodies (1:1,000 dilution) for 1 h at room temperature. Nuclei were stained with DAPI (10  $\mu$ g/ml) for 10 min. Samples were examined with Confocal Laser Scanning Microscopy (Zeiss) to analyze expression of E-cadherin, N-cadherin, fibronectin, ZO-1 and nuclear translocation of Snail.

## Immunoprecipitation

The cells were washed three times with ice-cold PBS and harvested at 4°C in immunoprecipitation lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, 10% glycerol, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin and 1  $\mu$ g/ml aprotinin. Equal amounts of protein were immunoprecipitated using anti-Snail or anti-GSK-3 $\beta$  antibody, and the immune complexes were bound to protein A/G Sepharose. The beads were washed with lysis buffer and subjected to western blotting with anti-ubiquitin, anti-Snail or anti-GSK-3 $\beta$  antibody.

## Statistical Analysis

Results were expressed as Mean  $\pm$  SD of three independent experiments unless otherwise specified. Data were analyzed by two-tailed unpaired Student's *t*-test between any two groups. One-way ANOVA analysis of variance was used to assess the difference of means among groups. These analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software Inc., La Jolla, CA). A *P*-value of <0.05 was considered statistically significant.

## Results

### TNF $\alpha$ Promotes Migration and Invasion of HCT116 Cells

Tumor cells with an aggressive phenotype acquire migratory and invasive capabilities. This will promote the dissemination of tumor cells to distant organs [8]. The migration and invasion abilities of HCT116 cells affected by TNF $\alpha$  were measured by using transwell migration and invasion assays. As shown in Figure 1A and C, TNF $\alpha$  treatment resulted in a significant increase in cell migration and invasion. Compared with control, the number of migrated and invasive cells increased about 4-fold (migration) and 20-fold (invasion) after treatment with TNF $\alpha$  (Fig. 1B and D).

### TNF $\alpha$ Induces EMT in HCT116 Cells

The increased migration and invasion abilities of tumor cells are reminiscent of the events at EMT, during which, the epithelial makers E-cadherin and ZO-1 are down-regulated, whereas the mesenchymal markers N-cadherin and fibronectin are up-regulated [27]. The EMT of HCT116 cells was observed after stimulation with 20 ng/ml TNF $\alpha$  for 4 days. Cells resulted in a significant change in morphology, from cobblestone morphology to mesenchymal spindle-like and fusiform features (Fig. 2A). Immunofluorescence analysis showed that this morphological change was associated with the down regulation of epithelial

characteristics E-cadherin, ZO-1 expression and the upregulation of mesenchymal characteristics fibronectin and N-cadherin expression (Fig. 2A). Similarly, western blotting analysis further confirmed the increasing expression of fibronectin and N-cadherin, and the decreasing expression of E-cadherin and ZO-1 at protein levels (Fig. 2B). Furthermore, qRT-PCR analysis showed that TNF $\alpha$  treatment down-regulated E-cadherin and up-regulated N-cadherin at mRNA levels (Fig. 2C). Collectively, these observations suggested that HCT116 cells had undergone an EMT after treated by TNF $\alpha$ .

### Snail is Crucial for TNF $\alpha$ -mediated EMT

Since transcription factors Snail, ZEB1, Twist and Slug play essential roles in regulating EMT [14], we then investigated whether their expressions were up-regulated in HCT116 cells after treated with TNF $\alpha$ . Compared to untreated cells, TNF $\alpha$  significantly increased Snail protein level, but not mRNA level (Fig. 2B and C). However, TNF $\alpha$  treatment altered neither mRNA nor protein levels of ZEB1, Twist, Slug (Fig. 2B and C).

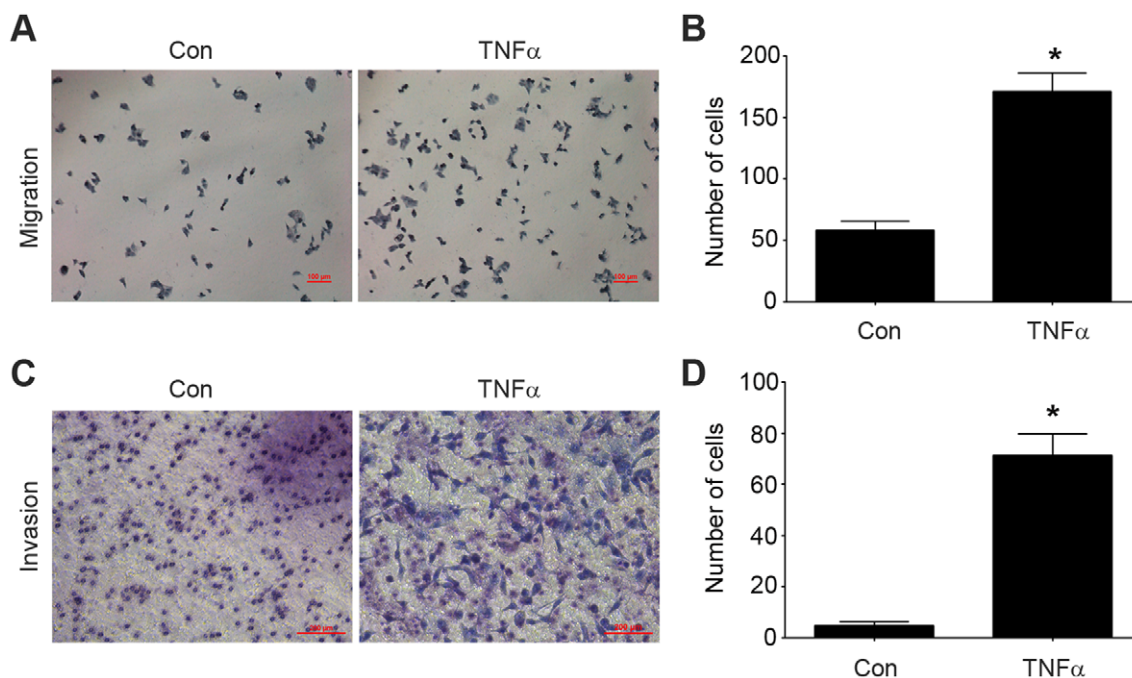
We overexpressed Snail to further investigate its roles in TNF $\alpha$ -induced EMT of HCT116 cells. Cells were transfected with pcDNA-Snail and control vector pcDNA-3.1, respectively. Expression of Snail and EMT markers were detected by immunofluorescence and western blotting. The results revealed that increased Snail expression induced EMT-like morphological changes and caused a switch from E-cadherin to N-cadherin expression in HCT116 cells (Fig. 3A and B). These findings demonstrated that ectopic expression of Snail can trigger EMT in HCT116 cells. Based on these observations, we assessed that Snail up-regulation may be crucial for TNF $\alpha$ -induced EMT in HCT116 cells.

We further performed knockdown assays to verify that Snail is a key regulator in TNF $\alpha$ -induced EMT. HCT116 cells were transfected with non-targeting control si-RNA or si-Snail for 24 h, and then treated with TNF $\alpha$  for different time. Morphological changes were observed under a phase contrast microscope. The expressions of Snail and E-cadherin were detected by western blotting. Compared to the control group, the spindle-like morphological changes were not observed upon TNF $\alpha$  addition in si-Snail transfected cells (Fig. 3C). Silencing of Snail also attenuated TNF $\alpha$ -induced down-regulation of E-cadherin, which was not observed in control si-RNA-transfected cells (Fig. 3D). Taken together, these observations demonstrated that Snail is essential for TNF $\alpha$ -induced EMT in HCT116 cells.

### TNF $\alpha$ Regulates Stabilization and Subcellular Localization of Snail

We previously found that TNF $\alpha$  increased Snail protein level, but not mRNA level (Fig. 2B and C). This result suggested that up-regulation of Snail by TNF $\alpha$  was occurring at the post-transcriptional level. To further verify this view, HCT116 and Caco-2 cells were treated with TNF $\alpha$  for 0–8 h, and Snail protein and mRNA were detected by western blotting and qRT-PCR, respectively. The results showed that the protein level of Snail was enhanced after 1 h of TNF $\alpha$  stimulation and increased time-dependently (Fig. 4A). However, the mRNA level of Snail did not have a significant change after TNF $\alpha$  treatment (Fig. 4B). These results suggested that the upregulation of Snail by TNF $\alpha$  might be due to protein stabilization.

Since transcription factors can only take effect when they transfer into the nucleus [28], we also determined the nuclear translocation activity of Snail by immunofluorescence. The nuclear localization of Snail was assessed in HCT116 cells stimulated with or without TNF $\alpha$  for 8 h. As shown in Fig. 4C,



**Figure 1. TNF $\alpha$  induces migration and invasion of HCT116 cells.** (A) HCT116 cells were allowed to migrate transwell chambers for 24 h in the presence or absence of TNF $\alpha$  (20 ng/ml). After 24 h, the migrated cells were fixed, stained, and photographed. Magnification, 100 $\times$ . (B) The number of migrated cells. Data represent the average of three independent experiments. (C) After treatment with or without TNF $\alpha$  (20 ng/ml) for 48 h, HCT116 cells that had spread through the matrixgel and into the under-side of the filter were fixed, stained, and photographed. Magnification, 200 $\times$ . (D) The number of invasive cells. Data represent the average of three independent experiments. \* $p < 0.05$  compared with control. doi:10.1371/journal.pone.0056664.g001

compared with the control group, TNF $\alpha$  significantly increased the nuclear translocation of Snail.

### TNF $\alpha$ Mediates Snail Stabilization via Activation of AKT and Inhibition of GSK-3 $\beta$

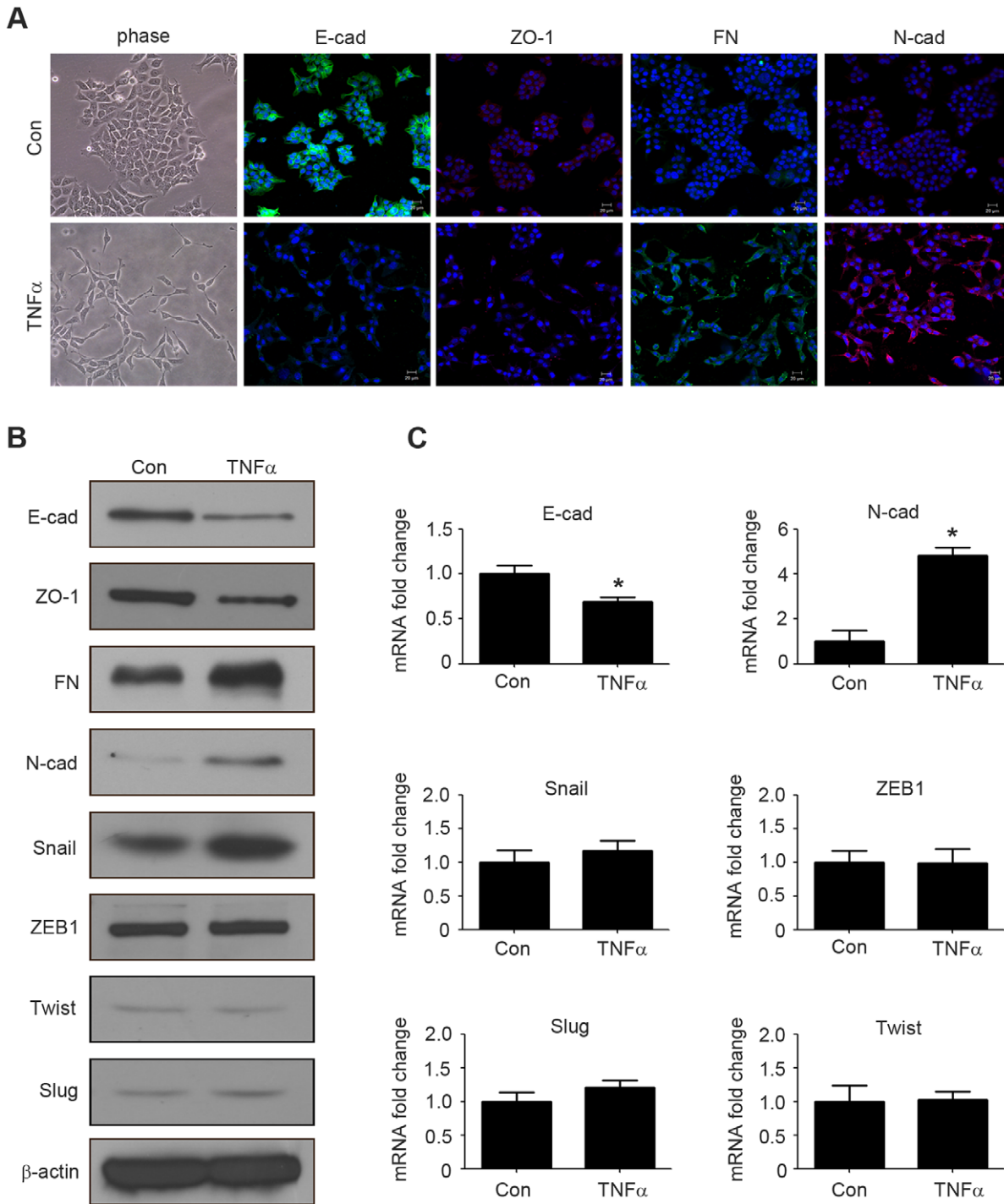
To investigate the molecular mechanisms underlying TNF $\alpha$ -mediated Snail stabilization, inhibitors of NF- $\kappa$ B (BAY11-7082), PI3K/AKT (LY294002), MAPK (PD98059), p38 (SB-203580) were used, since TNF $\alpha$  can induce the activation of these pathways. HCT116 and Caco-2 cells were pretreated with inhibitors for 1 h before TNF $\alpha$  stimulation, and then the expression of Snail was determined by western blotting. We found that PI3K/AKT inhibitor (LY294002), but not the others, completely blocked TNF $\alpha$ -stabilized Snail (Fig. 5A), suggesting that the activation of the PI3K/AKT pathway is responsible for TNF $\alpha$ -mediated Snail stabilization.

Snail is mainly regulated by GSK-3 $\beta$ , a kinase located downstream of the PI3K/AKT pathway [24,29,30]. GSK-3 $\beta$  maintains an active state in dephosphorylated form. To determine whether the stabilization of Snail by TNF $\alpha$  is mediated by regulation of GSK-3 $\beta$  activity, we treated HCT116 cells with LY294002 prior to TNF $\alpha$  treatment, and then the expression of p-AKT, p-GSK-3 $\beta$ , and Snail were determined by western blotting. We found that levels of p-AKT, p-GSK-3 $\beta$ , and Snail were increased after TNF $\alpha$  treatment at 6 h, while these effects were reversed upon treating with LY294002 alone or in combination with TNF $\alpha$  (Fig. 5B). We next measured the time courses for GSK-3 $\beta$  phosphorylation and found that the expression of p-GSK-3 $\beta$  increased in a time-dependent manner upon TNF $\alpha$  stimulation in HCT116 and Caco-2 cells (Fig. 5C). These results indicated that the stabilization of Snail by TNF $\alpha$  is due to the inhibition of the GSK-3 $\beta$  activity. To further confirm our findings,

we knocked down the expression of GSK-3 $\beta$  in HCT116 cells using specific GSK-3 $\beta$  si-RNA (Fig. 5D). Compared with control, down-regulation of GSK-3 $\beta$  markedly elevated the levels of Snail expression (Fig. 5D). However, TNF $\alpha$ -mediated Snail stabilization was not further elevated after knockdown of GSK-3 $\beta$  (Fig. 5D). Similarly, when we treated HCT116 cells with LiCl, a potent GSK-3 $\beta$  inhibitor, in accordance with TNF $\alpha$  treatment, the expressions of Snail, p-GSK-3 $\beta$ , and  $\beta$ -catenin (a protein regulated by GSK-3 $\beta$ ) were increased (Fig. 5E). However, the up-regulation of  $\beta$ -catenin was not as obvious as Snail. It might be due to intracellular localizations between  $\beta$ -catenin and Snail are different. To verify this hypothesis, we isolated Snail and  $\beta$ -catenin from membrane and nuclear of HCT116 cells treated with or without TNF $\alpha$ . The results revealed that different to Snail,  $\beta$ -catenin exists at the plasma membrane, and TNF $\alpha$  increases the nuclear translocation of Snail and  $\beta$ -catenin, but does not affect membrane  $\beta$ -catenin (Fig. 5F). Taken together, these results demonstrated that TNF $\alpha$  up-regulated Snail in HCT116 cells by activating AKT signaling that lead to the phosphorylation of GSK-3 $\beta$  and subsequently stabilize Snail.

### TNF $\alpha$ Suppresses Ubiquitylation of Snail by Inhibiting the Association of Snail and GSK-3 $\beta$

Because the protein stability of Snail is regulated via ubiquitin-mediated proteasomal degradation processes, we speculated whether the stabilization of Snail by TNF $\alpha$  is mediated by suppression of Snail ubiquitylation. To test this hypothesis, HCT116 cells were treated with TNF $\alpha$  or the proteasome inhibitor MG132 for 6 h, and then Snail was immunoprecipitated from equal amount of lysates. The ubiquitination state of Snail was detected by western blotting with an anti-ubiquitin antibody. The results revealed that compared with MG132, TNF $\alpha$  dramatically

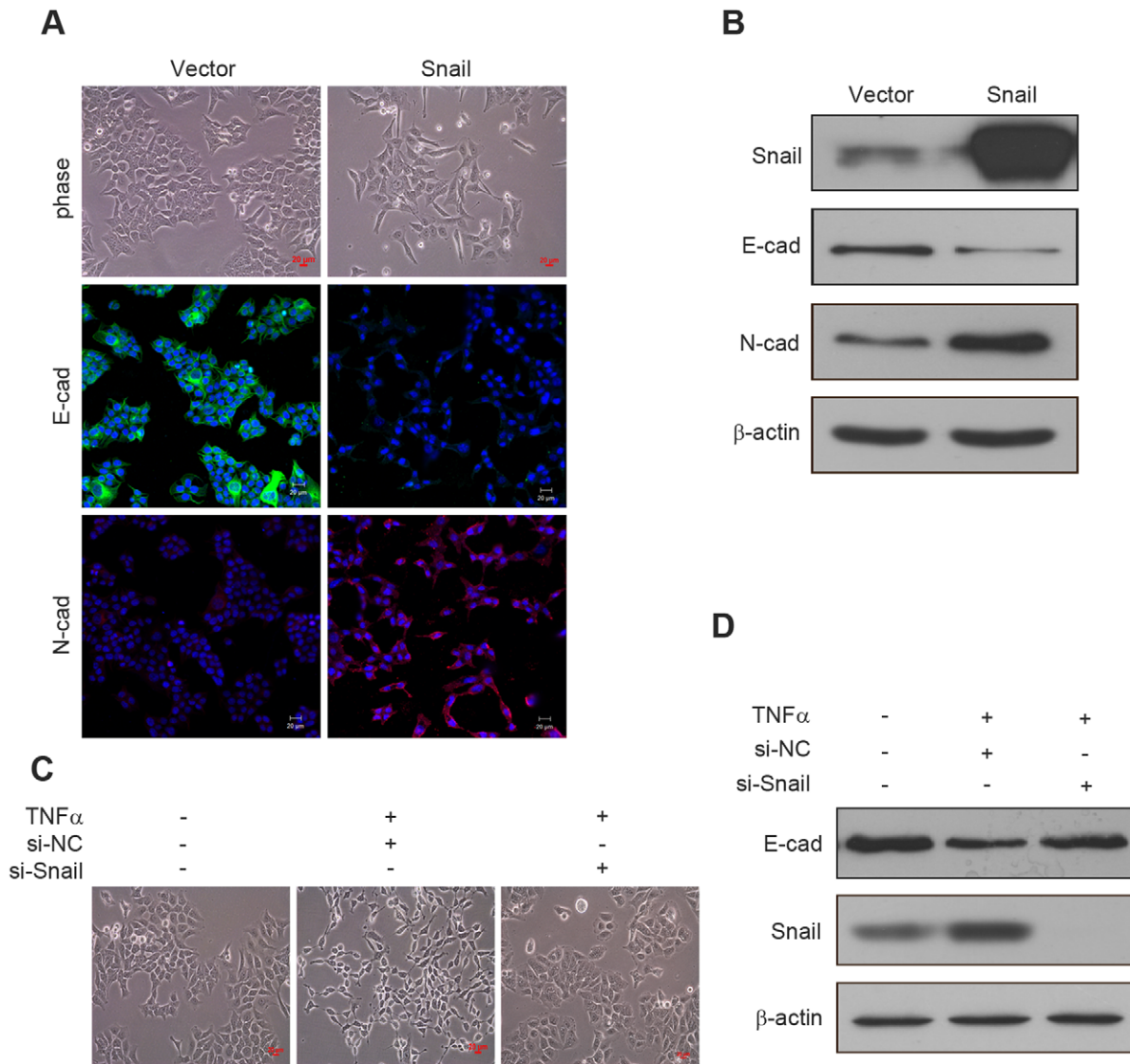


**Figure 2. TNF $\alpha$  treatment triggers EMT in HCT116 cells.** (A) HCT116 cells were treated with or without TNF $\alpha$  (20 ng/ml) for 4 days. Cell morphological changes associated with EMT are shown in the phase contrast image. Expression of E-cadherin, ZO-1, fibronectin, N-cadherin were analyzed by immunofluorescence staining. Nuclei were visualized with DAPI staining. Scale bars: 20  $\mu$ m. (B) HCT116 cells were treated with or without TNF $\alpha$  (20 ng/ml) for 4 days, and the expression of E-cadherin, ZO-1, fibronectin, N-cadherin, Snail, ZEB1, Twist, Slug were analyzed by western blotting.  $\beta$ -actin serves as the loading control. (C) HCT116 cells were treated with or without TNF $\alpha$  (20 ng/ml) for 4 days. The mRNA levels of E-cadherin, N-cadherin, Snail, ZEB1, Twist, Slug were analyzed by qRT-PCR. \* $p$ <0.05 compared with control. doi:10.1371/journal.pone.0056664.g002

suppressed the ubiquitylation of Snail, although total stabilized Snail proteins were parallel (Fig. 6A). Since GSK-3 $\beta$  is the main kinase that phosphorylates Snail and then induces the protein degradation of Snail [24], we next examined the association of

Snail and GSK-3 $\beta$ . HCT116 cells were treated with TNF $\alpha$  or MG132 for 6 h, and then Snail was immunoprecipitated from equal amount of lysates, and the associated GSK-3 $\beta$  was measured by western blotting. As shown in Fig. 6B, the association of Snail





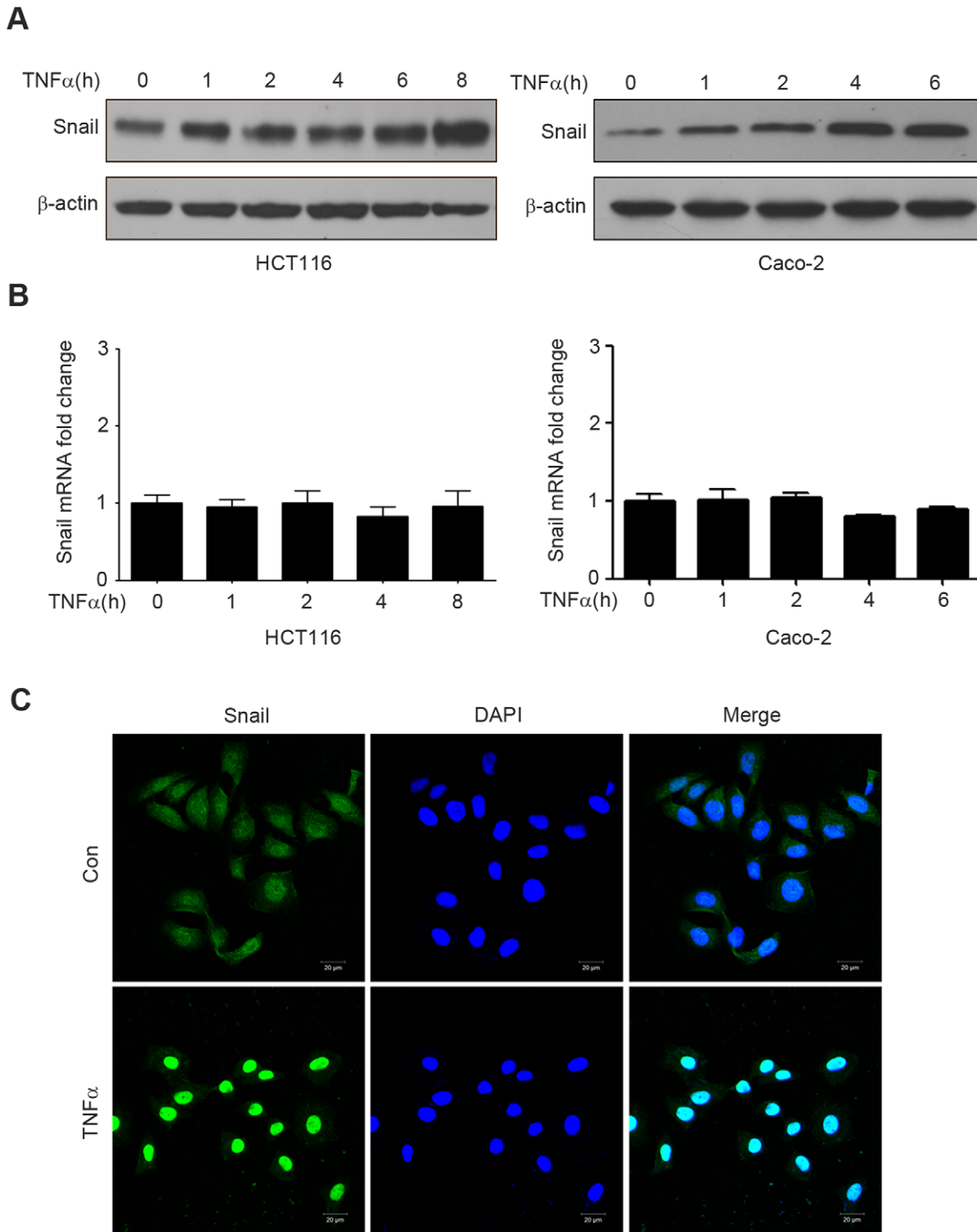
**Figure 3. Snail is crucial for TNF $\alpha$ -induced EMT.** (A) pcDNA-Snail (Snail) or control vector pcDNA-3.1 (Vector) were expressed in HCT116 cells for 48 h. Cell morphological changes associated with EMT are shown in the phase contrast image. Expression of E-cadherin and N-cadherin were analyzed by immunofluorescence staining. Nuclei were visualized with DAPI staining. Scale bars: 20  $\mu$ m. (B) The expression of E-cadherin, N-cadherin and Snail from HCT116 cells transfected with pcDNA-Snail or control vector were examined by western blotting.  $\beta$ -actin serves as the loading control. (C) HCT116 cells transfected with Snail specific si-RNA (si-Snail) or negative control si-RNA (si-NC) were stimulated with or without TNF $\alpha$  (20 ng/ml) for 48 h, and the morphologic changes were observed with a phase-contrast microscopy. (D) HCT116 cells transfected with si-Snail or si-NC were stimulated with or without TNF $\alpha$  (20 ng/ml) for 4 days, and the expression of E-cadherin and Snail were detected by western blotting.  $\beta$ -actin serves as the loading control.  
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with GSK-3 $\beta$  was diminished in cells treated with TNF $\alpha$ , compared with cells treated with MG-132. Similarly, when GSK-3 $\beta$  was immunoprecipitated from HCT116 cells, the associated Snail was markedly decreased in cells treated with TNF $\alpha$ , compared with cells treated with MG-132 (Fig. 6B). Taken together, these findings demonstrated that TNF $\alpha$  inhibited the association of Snail with GSK-3 $\beta$  and subsequently suppressed ubiquitylation of Snail.

**Discussion**

A major challenge during cancer therapy is metastasis induced by chronic inflammation. However, the underlying mechanisms are not entirely illustrated. Several inflammatory mediators, such

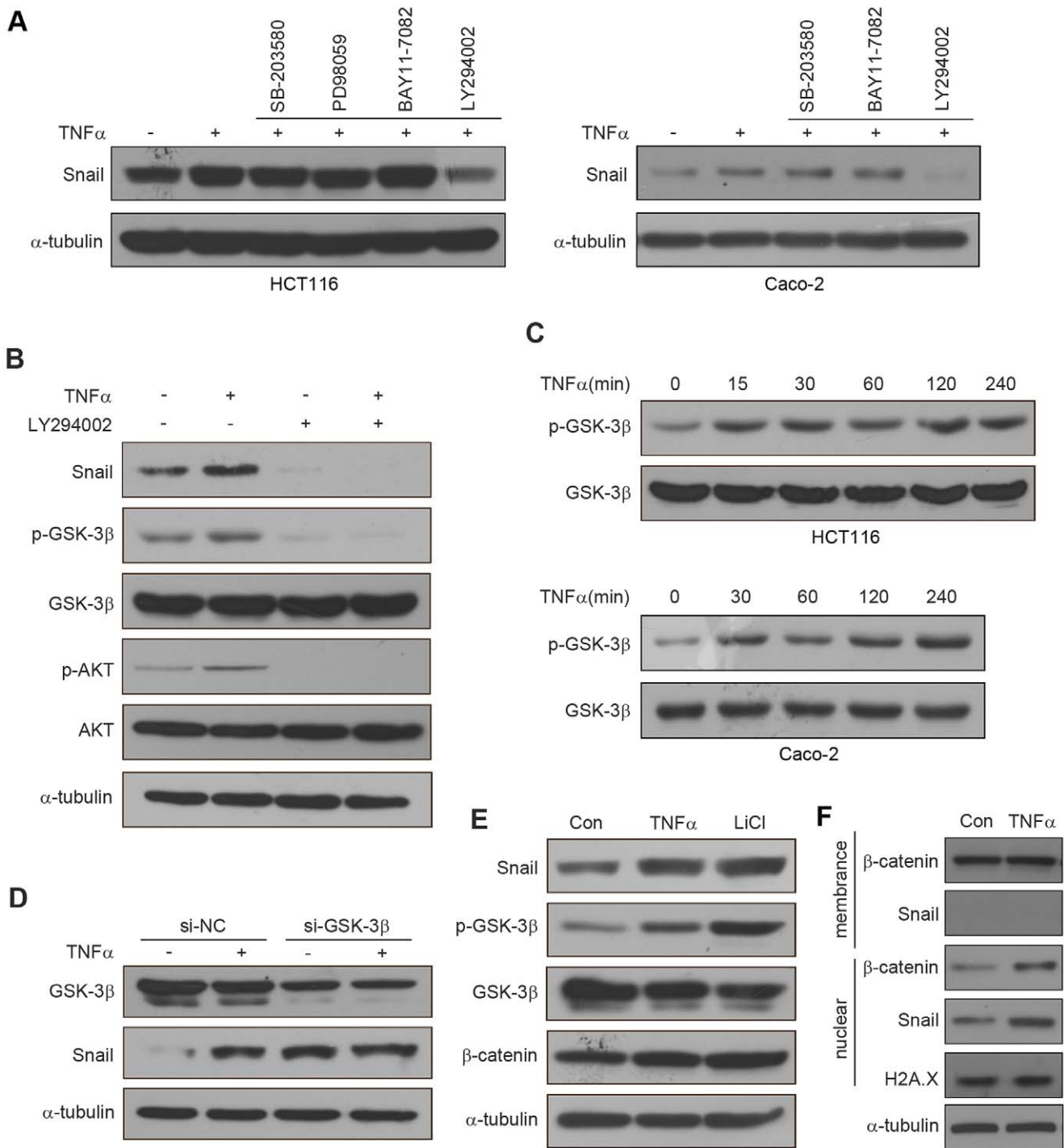
as TGF $\beta$  and IL-6, have been demonstrated that contribute to the invasion and metastasis of cancers [3]. TNF $\alpha$  is a major pro-inflammatory cytokine which has a wide range of biological activities, including inflammation, apoptosis, cell proliferation and differentiation [31]. Although TNF $\alpha$  has been considered as an anticancer agent, it is currently recognized that chronically elevated TNF $\alpha$  in tissues may promote tumor growth, invasion and metastasis [32]. There are some reports that TNF $\alpha$  expression is increased in the serum of CRC patients [33]. TNF $\alpha$  expression is also associated with tumor progression of colorectal adenocarcinomas [34]. In addition, High TNF $\alpha$  expression is strongly associated with tumor recurrence in CRC patients with positive lymph node metastase [34]. TNF $\alpha$  may be useful as a maker for the early diagnosis of CRC. In this study, we investigated an



**Figure 4. TNF $\alpha$  regulates Snail stabilization and localization.** (A–B) HCT116 and Caco-2 cells were treated with TNF $\alpha$  (20 ng/ml) for the times indicated, and the protein (A) and mRNA (B) levels of Snail were examined by western blotting and qRT-PCR respectively; (C) HCT116 cells were treated with or without TNF $\alpha$  (20 ng/ml) for 6 h. After fixation, the cellular location of Snail (green) was examined by immunofluorescence staining and nuclei were stained with DAPI (blue). Scale bars: 20  $\mu$ m.  
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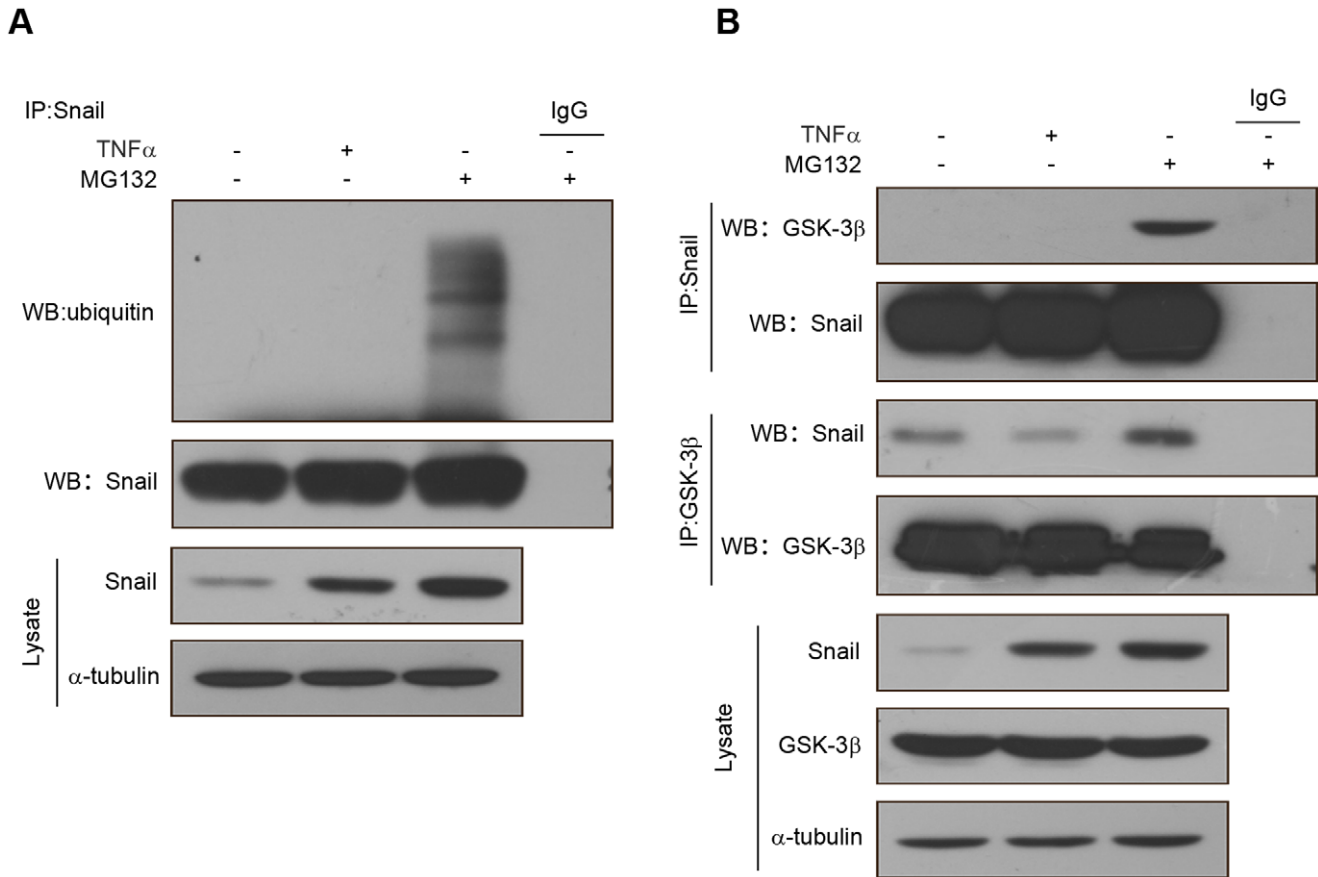
important signaling axis that controls inflammatory cytokines and induces EMT. Despite the essential role of NF- $\kappa$ B in inflammatory processes, we demonstrated that AKT/GSK-3 $\beta$ -mediated stabilization of Snail is required for TNF $\alpha$ -induced EMT in CRC cells.

Based on our findings, we proposed a model in which TNF $\alpha$  up-regulates Snail via activation of AKT signaling and thereby inhibits GSK-3 $\beta$  activity. TNF $\alpha$  also inhibits the association of Snail and GSK-3 $\beta$ . And then TNF $\alpha$  stabilized Snail transfer into



**Figure 5. TNF $\alpha$  stabilizes Snail through AKT/GSK3 $\beta$  pathway.** (A) HCT116 cells were pretreated with SB-203580 (20  $\mu$ M), PD98059 (20  $\mu$ M), BAY11-7082(10  $\mu$ M), LY294002 (20  $\mu$ M) for 1 h respectively followed by stimulation with TNF $\alpha$  (20 ng/ml) for 6 h. The expression of Snail was examined by western blotting. Caco-2 cells were pretreated with SB-203580 (20  $\mu$ M), BAY11-7082(10  $\mu$ M), LY294002 (20  $\mu$ M) for 1 h respectively followed by stimulation with TNF $\alpha$  (20 ng/ml) for 6 h. The expression of Snail was examined by western blotting. (B) HCT116 cells were pretreated with or without LY294002 (20  $\mu$ M) for 1 h, followed by stimulation with or without TNF $\alpha$  (20 ng/ml) for 6 h. The expression of Snail and the activation of AKT and GSK-3 $\beta$  were examined by western blotting. (C) HCT116 and Caco-2 cells were treated with TNF $\alpha$  (20 ng/ml) for the times indicated. The expression of pGSK-3 $\beta$  and GSK-3 $\beta$  were examined by western blotting. (D) Control and GSK-3 $\beta$  si-RNA were expressed in HCT116 cells for 42 h, followed treated with or without TNF $\alpha$  (20 ng/ml) for additional 6 h. The expression of Snail and GSK-3 $\beta$  were examined by western blotting. (E) HCT116 cells were treated with TNF $\alpha$  (20 ng/ml) or LiCl (40 mM) for 6 h. The expression of Snail, pGSK-3 $\beta$ , GSK-3 $\beta$ , and  $\beta$ -catenin were analyzed by western blotting. (F) After treated HCT116 cells with or without TNF $\alpha$  (20 ng/ml) for 6 h, Snail and  $\beta$ -catenin located at membrane and nuclear were isolated respectively and then analyzed by western blotting.  
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**Figure 6. TNF $\alpha$  inhibits the association of Snail and GSK3 $\beta$ .** (A) HCT116 cells were treated with TNF $\alpha$  (20 ng/ml) or MG132 (10  $\mu$ M) for 6 h. After Snail was immunoprecipitated from equal amount of lysates (two lower panels), the ubiquitination of Snail was examined by western blotting. (B) HCT116 cells were treated with TNF $\alpha$  (20 ng/ml) or MG132 (10  $\mu$ M) for 6 h. Snail or GSK-3 $\beta$  were immunoprecipitated respectively from equal amount of lysates and the associated GSK-3 $\beta$  or Snail were detected by western blotting. doi:10.1371/journal.pone.0056664.g006

the nucleus, decreases epithelial makers E-cadherin and ZO-1 expressions, increases mesenchymal makers N-cadherin and fibronectin expressions, finally induces EMT and promotes tumor metastasis.

EMT has been considered as the first step of tumor invasion and metastasis. Recent studies revealed that the expression profiles of EMT are correlated with tumor grades and metastasis of breast carcinoma [35,36]. Also, EMT plays a pivotal role in the metastasis of colon carcinoma, which occurs at the invasive front of colon carcinoma concomitant with a selective loss of basement membrane [37]. Snail is a zinc-finger transcription factor that has been known as an essential player in the aggressive phenotype of EMT [10]. In CRC tissues, Snail was highly expressed and the inverse correlation between Snail and E-cadherin was observed. Moreover, aberrant Snail expression correlated significantly with lymph node metastasis of CRC [38]. A recent study showed that TNF $\alpha$  induces EMT via up-regulation of Twist in breast cancer cells [39]. TNF $\alpha$  can also up-regulate Slug, which imparts breast cancer cells with a stem cell-like phenotype [40]. In this study, we detected the protein and mRNA levels of Snail, Slug, Twist and ZEB1 in HCT116 cells after exposure to TNF $\alpha$ . The protein level of Snail but not mRNA level exhibited a rapid response to TNF $\alpha$  stimulation. However, TNF $\alpha$  treatment altered neither mRNA nor protein levels of ZEB1, Slug and Twist. Overexpressed Snail also induces EMT, indicating that Snail may initiate EMT in TNF $\alpha$ -treated HCT116 cells. Accordingly, gene silencing of Snail

by si-RNA abolished TNF $\alpha$ -induced morphological changes and partly recovered the TNF $\alpha$  down regulated E-cadherin expression in HCT116 cells. These results suggested that there is a functional linkage between Snail expression and TNF $\alpha$ -mediated EMT in HCT116 cells. The knockdown of Snail did not fully reverse EMT induced by TNF $\alpha$ . Previous studies have demonstrated the function of many other transcription factors, such as ZEB2, E47, in controlling EMT during cancer progression [14]. It is worth to further investigating whether Snail can cooperate with the other transcription factors for the regulation of TNF $\alpha$ -mediated EMT in HCT116 cells.

Snail is a highly unstable protein with a short half-life and is regulated by a complex signaling network at both the transcriptional and post-transcriptional levels [41]. In this study, we demonstrated that TNF $\alpha$  stabilizes Snail via post-transcriptional regulation processes. A body of studies pointed out that NF- $\kappa$ B signaling is a crucial mediator linking between inflammation and cancer [42]. For example, NF- $\kappa$ B-mediated up-regulation of Twist is required for TNF $\alpha$ -induced EMT of breast cancer [39]. A recent study further demonstrated that inflammation induces invasion and metastasis via NF- $\kappa$ B-mediated stabilization of Snail [43]. They found that NF- $\kappa$ B inhibitor suppresses TNF $\alpha$ -stabilized Snail in Snail/HEK293 cells. However, our observations showed that in CRC cells NF- $\kappa$ B is not crucial for stabilization of Snail mediated by TNF $\alpha$ . The TNF $\alpha$ -enhanced Snail was blocked by treatment with PI3K inhibitor, but not NF- $\kappa$ B inhibitor,

suggesting that PI3K/AKT signaling pathway plays an essential role for TNF $\alpha$ -stabilized Snail of CRC cells. AKT pathway is frequently activated in various cancers and plays an critical role in promoting EMT and invasion [44–47]. For instance, activation of AKT pathway is required for induction of TGF $\beta$ - and EGF-dependent EMT [46]. AKT can also phosphorylate IKK $\alpha$  to increase Snail expression and induce EMT [48]. To date, GSK-3 $\beta$  has been characterized as a main kinase responsible for the subcellular location and protein stability of Snail [24,29,30]. In the present work, we found that activation of upstream AKT signaling represses GSK-3 $\beta$  activity. Also TNF $\alpha$  inhibits the association of Snail with GSK-3 $\beta$  and thereby increases the stability of Snail. After knockdown of GSK-3 $\beta$ , TNF $\alpha$ -mediated Snail stabilization is not further elevated.

Besides Snail, GSK-3 $\beta$  regulates stability of  $\beta$ -catenin by phosphorylating serine or threonine residues in its N-terminal domain [49]. In our study, we found that  $\beta$ -catenin was up-regulated by TNF $\alpha$  and LiCl (Figure 5E). However, this up-regulation was not as obvious as Snail. The reason may due to the different intracellular localizations between  $\beta$ -catenin and Snail. Though Snail and  $\beta$ -catenin localize in both the cytoplasm and the

nucleus,  $\beta$ -catenin acts as an important component of the action cytoskeleton at the plasma membrane [50]. Only cytosolic  $\beta$ -catenin can be phosphorylated by GSK-3 $\beta$  [49], so the alteration of  $\beta$ -catenin in whole cell lysate is not distinct with TNF $\alpha$  treatment.

In summary, we demonstrated that Snail plays a critical role in TNF $\alpha$ -induced EMT in HCT16 and Caco-2 cells. We also showed that TNF $\alpha$  up-regulates Snail by increasing its stability in two ways. On the one hand, TNF $\alpha$  activates AKT signaling and subsequently represses GSK-3 $\beta$  activity. On the other hand, TNF $\alpha$  inhibits the association of Snail with GSK-3 $\beta$ . These discoveries may provide a better understanding for colorectal cancer signaling and introduce potential therapeutic targets for malignant colorectal cancer.

### Author Contributions

Conceived and designed the experiments: HW. Performed the experiments: HW BHZ. Analyzed the data: BHZ SHC JD. Contributed reagents/materials/analysis tools: CLL FZ XFW GZ XZB. Wrote the paper: HW HSW SHC JD.

### References

- Coussens LM, Werb Z (2002) Inflammation and cancer. *Nature* 420: 860–867.
- Shacter E, Weitzman SA (2002) Chronic inflammation and cancer. *Oncology-New York* 16: 217–226.
- Wu Y, Zhou BP (2009) Inflammation a driving force speeds cancer metastasis. *Cell Cycle* 8: 3267–3273.
- Condeelis J, Pollard JW (2006) Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 124: 263–266.
- Burke F, Relf M, Negus R, Balkwill F (1996) A cytokine profile of normal and malignant ovary. *Cytokine* 8: 578–585.
- van Kempen L, Ruiter DJ, van Muijen GNP, Coussens LM (2003) The tumor microenvironment: a critical determinant of neoplastic evolution. *European Journal of Cell Biology* 82: 539–548.
- Balkwill F (2009) Tumour necrosis factor and cancer. *Nature Reviews Cancer* 9: 361–371.
- Thiery JP, Acloque H, Huang RYJ, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. *Cell* 139: 871–890.
- Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. *Journal of Clinical Investigation* 119: 1420–1428.
- Wu YD, Zhou BHP (2010) Snail more than EMT. *Cell Adhesion & Migration* 4: 199–203.
- Christofori G (2006) New signals from the invasive front. *Nature* 441: 444–450.
- Barrallo-Gimeno A, Nieto MA (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132: 3151–3161.
- Thiery JP, Sleeman JP (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nature Reviews Molecular Cell Biology* 7: 131–142.
- Peinado H, Olmeda D, Cano A (2007) Snail, ZEB and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nature Reviews Cancer* 7: 415–428.
- Nieto MA (2002) The snail superfamily of zinc-finger transcription factors. *Nature Reviews Molecular Cell Biology* 3: 155–166.
- Battle E, Sancho E, Franci C, Dominguez D, Monfar M, et al. (2000) The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nature Cell Biology* 2: 84–89.
- Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, et al. (2000) The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature Cell Biology* 2: 76–83.
- Dong CF, Wu YD, Yao J, Wang YF, Yu YH, et al. (2012) G9a interacts with Snail and is critical for Snail-mediated E-cadherin repression in human breast cancer. *Journal of Clinical Investigation* 122: 1469–1486.
- Pon YL, Zhou HY, Cheung ANY, Ngan HYS, Wong AST (2008) p70 S6 kinase promotes epithelial to mesenchymal transition through Snail induction in ovarian cancer cells. *Cancer Research* 68: 6524–6532.
- Olmeda D, Jorda M, Peinado H, Fabra A, Cano A (2007) Snail silencing effectively suppresses tumour growth and invasiveness. *Oncogene* 26: 1862–1874.
- Blanco MJ, Moreno-Bueno G, Sarrío D, Locascio A, Cano A, et al. (2002) Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* 21: 3241–3246.
- Cheng CW, Wu PE, Yu JC, Huang CS, Yue CT, et al. (2001) Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. *Oncogene* 20: 3814–3823.
- Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, et al. (2005) The transcriptional repressor snail promotes mammary tumor recurrence. *Cancer Cell* 8: 197–209.
- Zhou BHP, Deng J, Xia WY, Xu JH, Li YM, et al. (2004) Dual regulation of Snail by GSK-3 $\beta$  mediated phosphorylation in control of epithelial-mesenchymal transition. *Nature Cell Biology* 6: 931–940.
- Mak P, Leav I, Pursell B, Bae D, Yang XF, et al. (2010) ER beta impedes prostate cancer EMT by destabilizing HIF-1 $\alpha$  and inhibiting VEGF-mediated Snail nuclear localization: Implications for Gleason Grading. *Cancer Cell* 17: 319–332.
- Xiong H, Hong J, Du W, Lin YW, Ren LL, et al. (2012) Roles of STAT3 and ZEB1 proteins in E-cadherin down-regulation and human colorectal cancer epithelial-mesenchymal transition. *Journal of Biological Chemistry* 287: 5819–5832.
- Voulgari A, Pintzas A (2009) Epithelial-mesenchymal transition in cancer metastasis: Mechanisms, markers and strategies to overcome drug resistance in the clinic. *Biochimica Et Biophysica Acta-Reviews on Cancer* 1796: 75–90.
- Vandromme M, GauthierRouviere C, Lamb N, Fernandez A (1996) Regulation of transcription factor localization: fine-tuning of gene expression. *Trends in Biochemical Sciences* 21: 59–64.
- Bachelder RE, Yoon SO, Franci C, de Herreros AG, Mercurio AM (2005) Glycogen synthase kinase-3 is an endogenous inhibitor of snail transcription: implications for the epithelial-mesenchymal transition. *Journal of Cell Biology* 168: 29–33.
- Dominguez D, Montserrat-Sentis B, Virgos-Soler A, Guaita S, Grueso J, et al. (2003) Phosphorylation regulates the subcellular location and activity of the snail transcriptional repressor. *Molecular and Cellular Biology* 23: 5078–5089.
- Choo MK, Sakurai H, Koizumi K, Saiki I (2005) Stimulation of cultured colon 26 cells with TNF-alpha promotes lung metastasis through the extracellular signal-regulated kinase pathway. *Cancer Letters* 230: 47–56.
- Szlosarek P, Charles KA, Balkwill FR (2006) Tumour necrosis factor-alpha as a tumour promoter. *European Journal of Cancer* 42: 745–750.
- Csiszar A, Szentes T, Haraszti B, Balazs A, Petranyi GG, et al. (2004) The pattern of cytokine gene expression in human colorectal carcinoma. *Pathology & Oncology Research* 10: 109–116.
- Grimm M, Lazariotou M, Kircher S, Hofelmayr A, Germer CT, et al. (2011) Tumour necrosis factor-alpha is associated with positive lymph node status in patients with recurrence of colorectal cancer-indications for anti-TNF-alpha agents in cancer treatment. *Cellular Oncology* 34: 315–326.
- Logullo AF, Nonogaki S, Pasini FS, Osorio C, Soares FA, et al. (2010) Concomitant expression of epithelial-mesenchymal transition biomarkers in breast ductal carcinoma: Association with progression. *Oncology Reports* 23: 313–320.
- Xue CS, Pliehl D, Venkov C, Xu C, Neilson EG (2003) The gatekeeper effect of epithelial-mesenchymal transition regulates the frequency of breast cancer metastasis. *Cancer Research* 63: 3386–3394.
- Sheehan KM, Gulmann C, Eichler GS, Weinstein JN, Barrett HL, et al. (2008) Signal pathway pro. ling of epithelial and stromal compartments of colonic carcinoma reveals epithelial-mesenchymal transition. *Oncogene* 27: 323–331.
- Fan XJ, Wan XB, Yang ZL, Fu XH, Huang Y, et al. (2012) Snail promotes lymph node metastasis and Twist enhances tumor deposit formation through epithelial-mesenchymal transition in colorectal cancer. *Human Pathology*. DOI:10.1016/j.humpath.2012.03.029.

39. Li CW, Xia WY, Huo LF, Lim SO, Wu Y, et al. (2012) Epithelial-mesenchymal transition induced by TNF-alpha requires NF-kappa B-mediated transcriptional upregulation of Twist1. *Cancer Research* 72: 1290–1300.
40. Storci G, Sansone P, Mari S, D'Uva G, Tavorari S, et al. (2010) TNFalpha up-regulates Slug via the NF-kappaB/HIF1alpha axis, which imparts breast cancer cells with a stem cell-like phenotype. *Journal of Cellular Physiology* 225: 682–691.
41. De Craene B, van Roy F, Berx G (2005) Unraveling signalling cascades for the Snail family of transcription factors. *Cellular Signalling* 17: 535–547.
42. Karin M, Greten FR (2005) NF kappa B: linking inflammation and immunity to cancer development and progression. *Nature Reviews Immunology* 5: 749–759.
43. Wu Y, Deng J, Rychahou PG, Qiu SM, Evers BM, et al. (2009) Stabilization of Snail by NF-kappa B is required for inflammation-induced cell migration and invasion. *Cancer Cell* 15: 416–428.
44. Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, van Roy F, et al. (2003) The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. *Cancer Research* 63: 2172–2178.
45. Saegusa M, Hashimura M, Kuwata T, Okayasu I (2009) Requirement of the Akt/beta-catenin pathway for uterine carcinosarcoma genesis, modulating E-cadherin expression through the transactivation of Slug. *American Journal of Pathology* 174: 2107–2115.
46. Larue L, Bellacosa A (2005) Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene* 24: 7443–7454.
47. Chen RQ, Yang QK, Lee JD (2012) BMK1 kinase suppresses epithelial-mesenchymal transition through the Akt/GSK3 beta signaling pathway. *Cancer Research* 72: 1579–1587.
48. Julien S, Puig I, Caretti E, Bonaventure J, Nelles L, et al. (2007) Activation of NF-kappa B by Akt upregulates Snail expression and induces epithelium mesenchyme transition. *Oncogene* 26: 7445–7456.
49. Liu CM, Li YM, Semenov M, Han C, Baeg GH, et al. (2002) Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* 108: 837–847.
50. Schmalhofer O, Brabletz S, Brabletz T (2009) E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. *Cancer and Metastasis Reviews* 28: 151–166.