

Exposure to TNF- α combined with TGF- β induces carcinogenesis *in vitro* via NF- κ B/Twist axis

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Received August 22, 2016; Accepted January 3, 2017

DOI: 10.3892/or.2017.5369

Abstract. Persistent human papilloma virus (HPV) infection induces chronic inflammation resulting in human cervical cancer. However, the mechanisms underlying carcinogenesis via chronic inflammation remain largely unclear. We investigated the role of pro-inflammatory factors in epithelial-mesenchymal transition (EMT) and cancer stem cell-like (CSCL) characteristics of HeLa cells exposed to TNF- α with or without TGF- β . We then determined the role of NF- κ B/Twist signal axis in the pathogenesis of cervical cancer. We found that HeLa cells exposed to TNF- α following chronic treatment with TGF- β exhibited EMT, self-renewal and high mobility. Knockdown of NF- κ Bp65 inhibited NF- κ B and Twist1 expression, and EMT and CSCL properties of HeLa cells following co-treatment with TNF- α and TGF- β . Conversely, overexpression of NF- κ Bp65 potentiated the above effects. However, knockdown or overexpression of Twist1 had no effect on NF- κ Bp65 expression, but inhibited or promoted EMT and CSCL features. Notably, overexpression of Twist1 rescued NF- κ Bp65 knockdown. Our results demonstrate the role of NF- κ B/Twist signaling axis in which HeLa cells treated with TNF- α following chronic exposure to TGF- β induce EMT and CSCL properties. The NF- κ B/Twist signal axis may represent an effective therapeutic target in cervical cancer.

Introduction

Cervical cancer is a key factor associated with morbidity and mortality in women worldwide (1). Infection with human papilloma viruses (HPV) triggers carcinogenesis. Most of the precancerous lesions do not progress to invasive carcinoma, suggesting that HPV is not the only factor contributing to the development of cervical cancer (2,3). However, persistent HPV infection alters the pro-inflammatory cytokine profile, resulting in chronic inflammation and recurrence of cervical cancer (4). Cancer stem cells (CSCs) play a vital role in cancer initiation and metastasis (5). Metastasis results in treatment failure and death (6). Epithelial-mesenchymal transition (EMT) has been implicated as the key factor in CSCs transformation (7,8). EMT has been shown to induce reversion to a CSC-like phenotype, linking CSCs and EMT (9,10).

NF- κ B is a classic transcription factor activated by inflammatory stimuli, such as LPS (11), TNF- α (12) and IL-10 (13). Activated NF- κ B induces extensive gene expression in immune response (TNF- α), angiogenesis (VEGF), invasion (MMP-9) and EMT (Twist) (14-17). Furthermore, NF- κ B, a pleiotropic transcription factor, has been implicated in EMT and metastasis (14-17). In mammary epithelial cells, EMT is upregulated via overexpression of NF- κ Bp65 (17).

The transcriptional factor TWIST mediates EMT and cancer metastasis (18,19). In uterine cancers, *Twist* overexpression promotes invasion and metastasis (20-23). However, the role of NF- κ B/Twist axis in cervical cancer has not been investigated. In this study, we focused on the role of NF- κ B/Twist axis *in vitro*, by co-treatment of human cervical cancer cell line HeLa with TNF- α and TGF- β .

Materials and methods

Reagents. DMEM was obtained from Gibco, FBS from PAA, trypsin and penicillin-streptomycin from Invitrogen, and TGF- β and TNF- α from Sino Biological (Beijing, China). Anti-E-cadherin and anti-N-cadherin antibodies were supplied by Cell Signaling Technology (Danvers, MA,

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Abbreviations: CSCL, cancer stem cell-like; CSCs, cancer stem cells; EMT, epithelial-mesenchymal transition; HPV, persistent human papilloma virus; HRP, horseradish peroxidase

Key words: cervical cancer, cancer stem cell, epithelial-mesenchymal transition, NF- κ B, Twist

USA). The following antibodies were purchased from Abcam (Burlingame, CA, USA): anti-Bmi1, anti-Sox2, anti-Oct4, anti-CD133, anti-CD44, anti-ALDH1, anti-NF- κ Bp65 and anti-Twist1.

Cell culture and EMT morphology. HeLa cells were supplied by the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured as described by López *et al* (5). Cells were incubated with TNF- α (10.0 ng/ml) for 24 h, TGF- β (5.0 ng/ml) for 6 days or TNF- α (10.0 ng/ml) for 24 h along with TGF- β (5.0 ng/ml) for 6 days. EMT morphology were visualized under a light microscope (Olympus, Japan). The regulation of gene expression was studied by transducing cells with either NF- κ Bp65 shRNA and Twist1 shRNA or NF- κ Bp65 and Twist1-carrying adenoviruses obtained from Hanbio Biothechnology Co. Ltd. (1.0 ml, 1×10^{11} pfu/ml; Shanghai, China).

Wound healing assay. Wound healing was tested by loading cells (5×10^5) in 6-well plates and grown until cells attained 90% confluence. A scratch was created using a 100- μ l pipette tip and rinsed with PBS. Photographs were obtained and analyzed at 24 h, and the migrating cell number was standardized with mock.

Sphere formation. Cells (1,000 cells/ml) were loaded on ultra-low attachment 24-well culture plates (Corning, USA) in stem cell conditional medium. Five days later, the number of spheroids in each well was scored. The sphere formation rate was calculated as a percentage of the total number of spheres among the viable cells.

Western blot analysis. Whole cell lysates were prepared as previously described (24). The primary antibodies used for membrane incubation were as follows: anti-E-cadherin, anti-N-cadherin, anti-Bmi1, anti-Sox2, anti-Oct4, anti-CD133, anti-CD44, anti-ALDH1, anti-NF- κ B and anti-Twist. The membranes were further incubated with anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP). After incubation, the specific protein bands were visualized by enhanced chemiluminescence, using β -actin as a loading control.

Statistical analysis. Experimental data were analyzed using SPSS 20.0 for Windows (SPSS Inc, Chicago, IL, USA). Data representing mean \pm SD were subjected to one-way ANOVA. First, the homogeneity of variance was determined. We used LSD to analyze pairwise comparisons among the groups. In the event of incomplete variance, the control and the experimental groups were analyzed with Tukey's test. A probability of <0.05 suggested statistical significance.

Results

Treatment with TNF- α or TGF- β or both induces EMT and CSCL properties in HeLa cells and increases NF- κ B and Twist levels. TNF- α induces proliferation of epithelial tumor cells following exposure to TGF- β and EMT (17,19). Morphological features ranging from cobblestone appearance to spindle phenotypes were observed in HeLa cells after exposure to pro-inflammatory cytokines TGF- β (5.0 ng/ml) or

TNF- α (10.0 ng/ml) or both (Fig. 1A). Western blot analysis results were validated using antibodies targeting EMT-related markers. As shown in Fig. 1B, the pro-inflammatory cytokines downregulated E-cadherin and upregulated N-cadherin expression. Concurrently, we measured the cell migration and self-renewal after treatment with the inflammatory cytokines. The results showed that the combination of pro-inflammatory cytokines enhanced migration (Fig. 1C) and self-renewal (Fig. 1D) of HeLa cells. The multipotent stem cell factors Bmi1, Sox2 and Oct4 were overexpressed (Fig. 1E). Similarly, the stem cell markers CD133, CD44 and ALDH1 were upregulated (Fig. 1F). NF- κ B is a key regulator of inflammation, and Twist plays an important role in EMT (17,25,26). As illustrated in Fig. 1G, Western blots revealed an overexpression of NF- κ B and Twist1 in HeLa cells after treatment with the different cytokines, either alone or in combination.

In this preliminary experiment, the characteristic EMT phenotype was apparent in HeLa cells exposed to TNF- α alone for 24 h, and combined with TGF- β for 6 days (data not shown).

Silencing of NF- κ Bp65 downregulates Twist and reverses EMT and CSCL features in HeLa cells exposed to inflammatory cytokines. The expression of NF- κ Bp65 and Twist in shNF- κ Bp65-expressing HeLa cells exposed to TGF- β and TNF- α was significantly lower than in the control cells (Fig. 2A). EMT morphological changes and relevant protein expression were detected. As shown in Fig. 2B and C, NF- κ Bp65 shRNA-expressing HeLa cells exhibited cobblestone-like morphology, while GFP control cells displayed spindle shape. Furthermore, NF- κ Bp65 shRNA-expressing HeLa cells expressed higher levels of E-cadherin in epithelial cells, and a lower level of N-cadherin. The wound healing and sphere formation assays revealed that silencing of NF- κ Bp65 expression in HeLa cells decreased migration and self-renewal following co-treatment with TNF- α and TGF- β (Fig. 2D and E). Furthermore, compared with control cells, knockdown of NF- κ Bp65 expression reduced the expression of multi-functional proteins Bmi1, Sox2 and Oct4 (Fig. 2F), while CSC surface markers CD133, CD44 and ALDH1 (Fig. 2G) were induced by co-treatment with TNF- α and chronic exposure to TGF- β .

Overexpression of NF- κ Bp65 upregulates Twist and promotes EMT and CSCL properties in HeLa cells exposed to inflammatory cytokines. We evaluated the effect of overexpression of NF- κ Bp65 on EMT and CSCL properties of HeLa cells exposed to TNF- α and TGF- β . NF- κ Bp65-expressing adenovirus-infected HeLa cells overexpressed NF- κ Bp65 and Twist1 (Fig. 3A). As illustrated in Fig. 3B and C, increased expression of mesenchymal marker N-cadherin and decreased expression of epithelial marker E-cadherin and spindle shape were found in NF- κ Bp65-expressing HeLa cells. These findings suggested that NF- κ B mediated a significant switch from epithelial to mesenchymal phenotypes in HeLa cells following exposure to TNF- α and TGF- β . As shown in Fig. 3D and E, NF- κ Bp65 overexpression results in increased cell migration and self-renewal in HeLa cells. Furthermore, we found that NF- κ Bp65 expression modulated Bmi1, Sox2, Oct4 (Fig. 3F), and CD133, CD44 and ALDH1 (Fig. 3G) in HeLa cells exposed to proinflammatory cytokines.

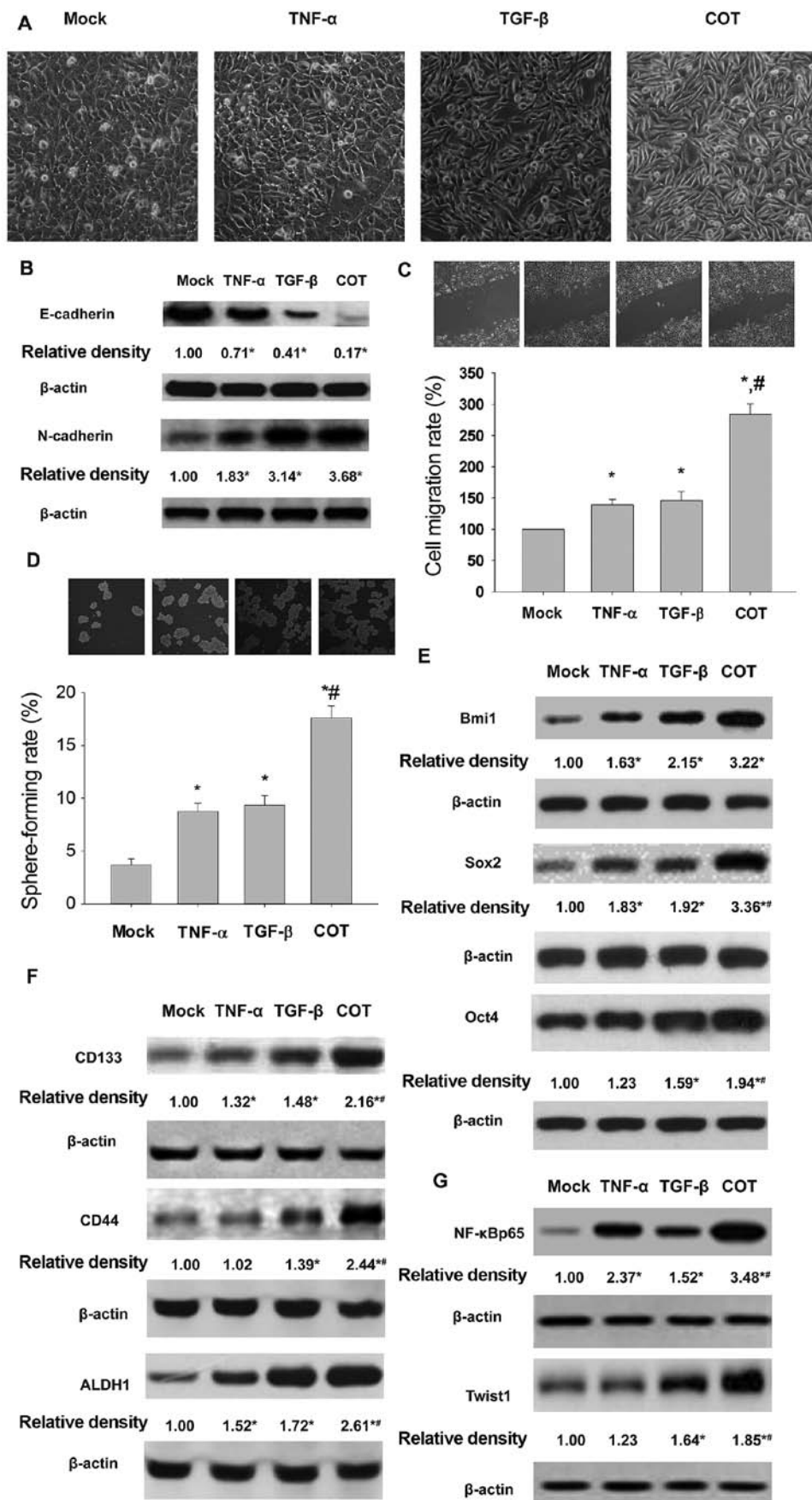


Figure 1. Treatment with TGF- β and TNF- α alone or in combination contributes to EMT and CSC properties of HeLa cells. HeLa cells exposed to TNF- α and TGF- β display mesenchymal morphology (x20) (A), E-cadherin and N-cadherin expression (B), cell migration (C), self-renewal (D), expression of Bmi1, Sox2 and Oct4 (E), CD133, ALDH1 and CD44 (F) and NF- κ B, and Twist1 proteins. Mock, cells exposed to complete medium; TNF- α , cells exposed to 10.0 ng/ml of TNF- α for 24 h; TGF- β , cells were exposed to 5.0 ng/ml of TGF- β for 6 days; COT, cells were exposed to 10.0 ng/ml TNF- α for 24 h and 5.0 ng/ml of TGF- β for 6 days. *P<0.05, vs mock; **P<0.05, vs TNF- α or TGF- β .

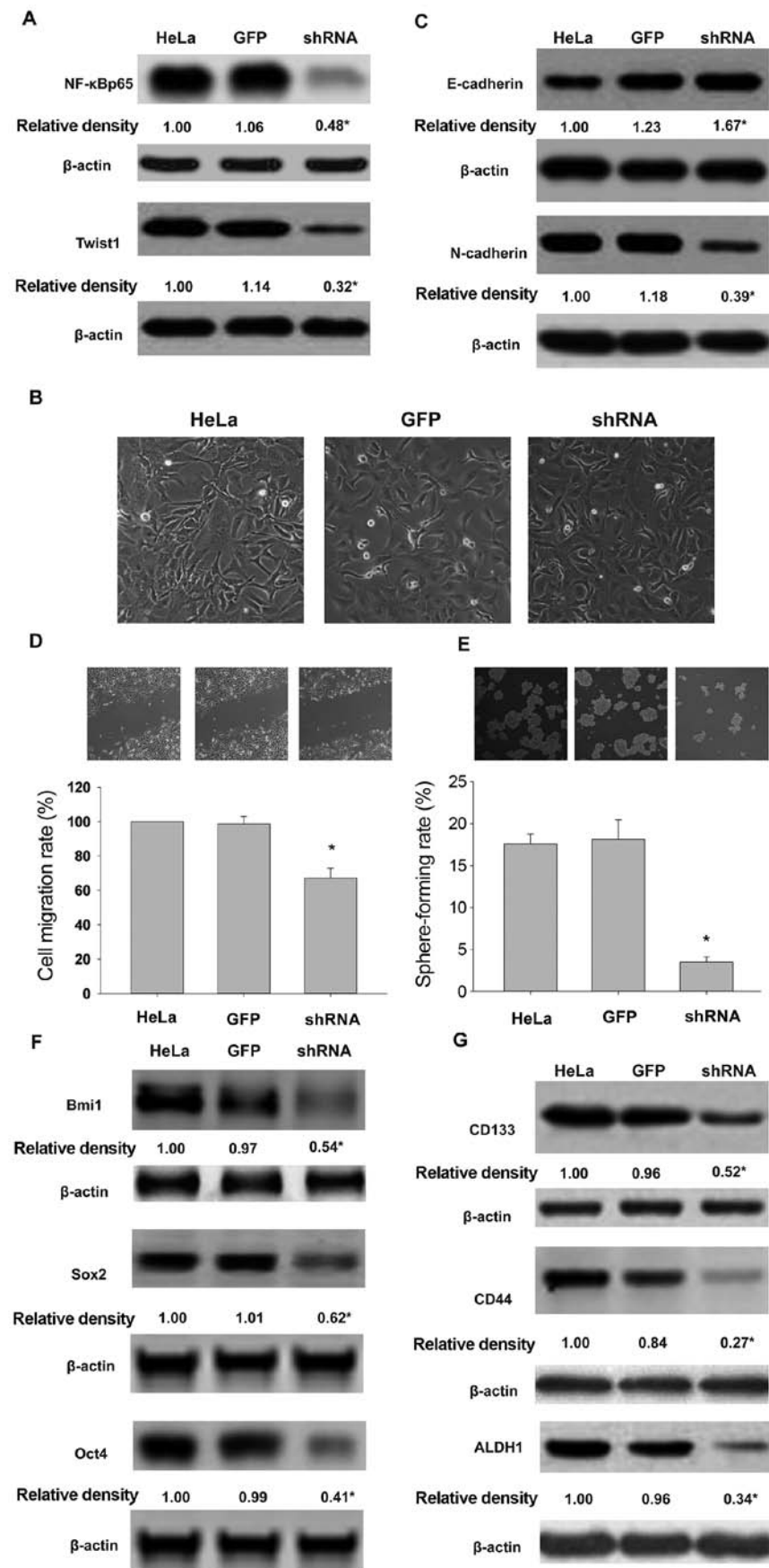


Figure 2. Silencing of NF- κ Bp65 leads to Twist1 downregulation and attenuates the effect of TNF- α combined with TGF- β on EMT and CSCL properties of HeLa cells. NF- κ Bp65 silencing affects the expression of NF- κ Bp65 and Twist1 proteins (A), EMT morphology (B), E-cadherin and N-cadherin profile (C), cell migration (D), sphere formation (E), expression of Bmi1, Sox2 and Oct4 (F), and CD133, CD44 and ALDH1 (G) in HeLa cells exposed to inflammatory cytokines. HeLa, HeLa cells exposed to TNF- α and TGF- β . GFP, cells transduced with adenovirus expressing GFP. shRNA, cells transduced with adenovirus expressing shNF- κ Bp65. *P<0.05, vs. HeLa or GFP.

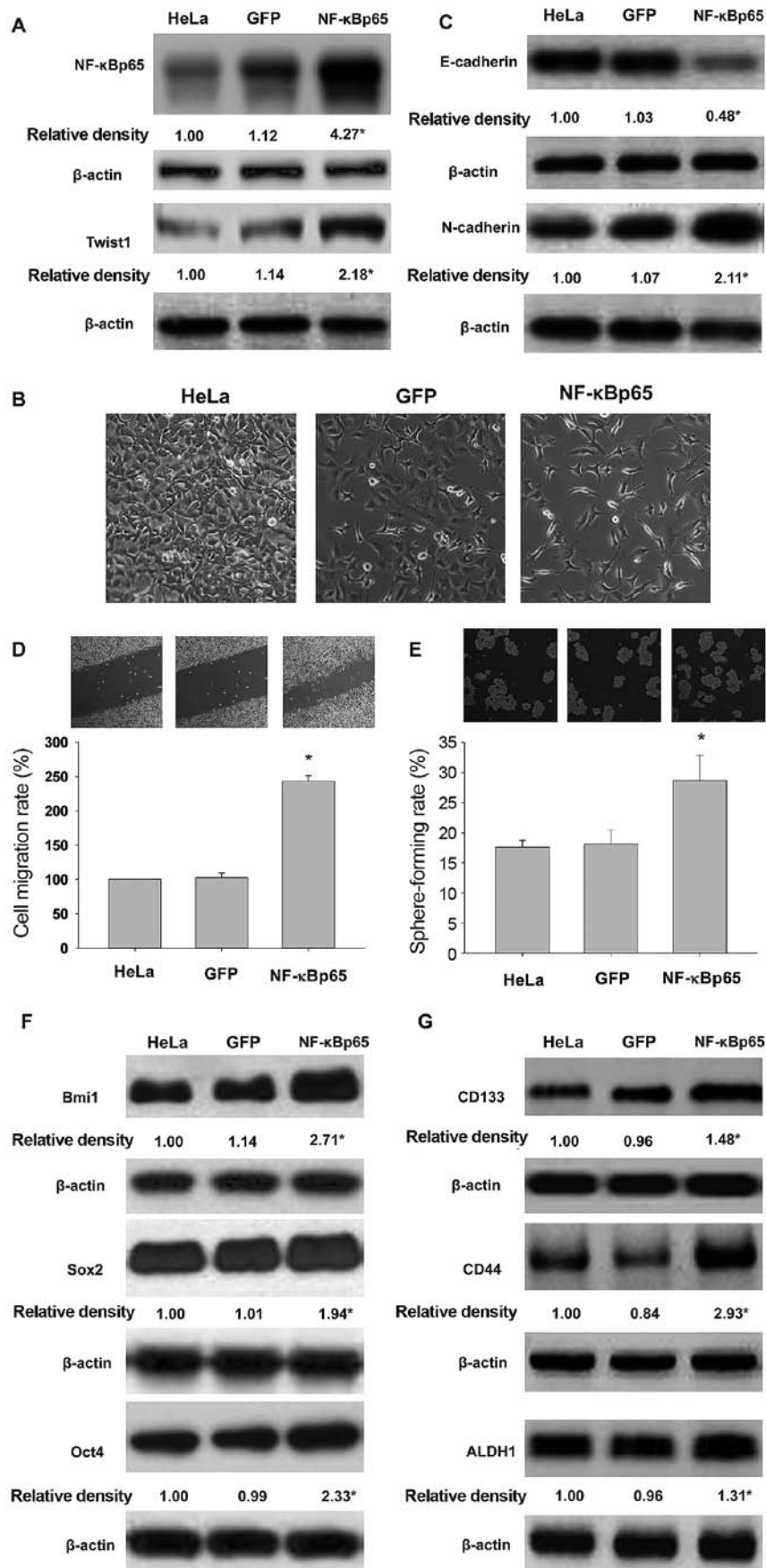


Figure 3. Overexpression of NF-κBp65 increases the effects of exposure to TNF-α and TGF-β on EMT and CSCL properties in HeLa cells. Effects of *NF-κBp65* gene transduction on NF-κBp65 and Twist1 protein levels (A), EMT morphology (B), E-cadherin and N-cadherin profile (C), cell migration (D), sphere formation (E), Bmi1, Sox2 and Oct4 levels (F) and the expression of CD133, CD44 and ALDH1 (G) in HeLa cells exposed to TNF-α and TGF-β. HeLa, HeLa cells exposed to TNF-α and TGF-β. GFP, cells transduced with *GFP* gene; NF-κBp65, cells transduced with *NF-κBp65* gene. *P<0.05, vs HeLa or GFP.

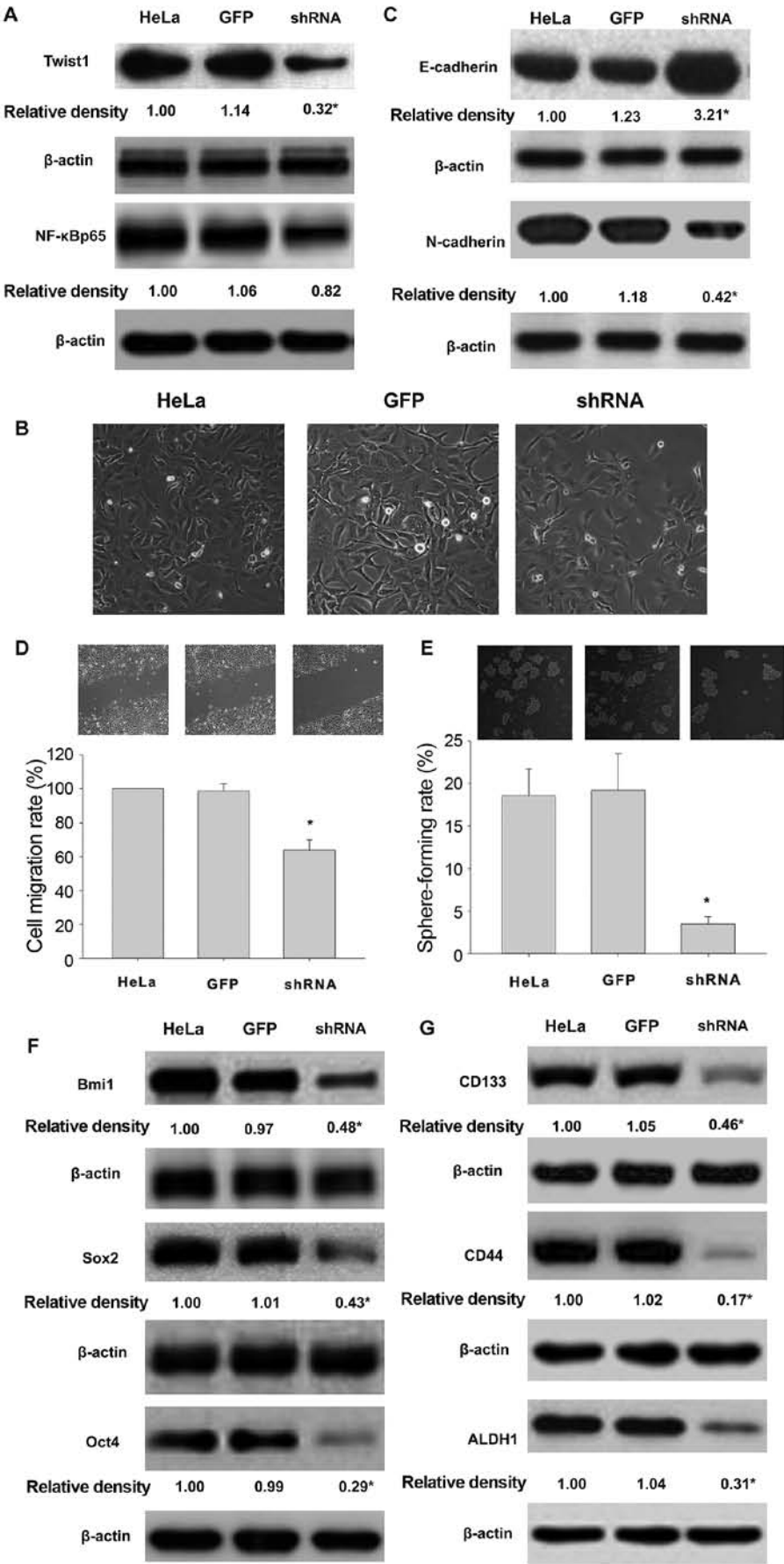


Figure 4. Knockdown of Twist1 decreases the impact of exposure to TGF- β and TNF- α on EMT and CSCL properties of HeLa cells. Effects of Twist1 silencing on the expression of Twist1 and NF- κ Bp65 (A), EMT morphology (B), E-cadherin and N-cadherin profile (C) cell migration (D), sphere formation (E), expression of Bmi1, Sox2 and Oct4 (F) and CD133, CD44 and ALDH1 (G) in HeLa cells exposed to TNF- α and TGF- β . HeLa, HeLa cells exposed to TNF- α and TGF- β . GFP, cells transduced with *GFP* gene. shRNA, cells transduced with sh Twist1 (* P <0.05). * P <0.05, vs HeLa or GFP.

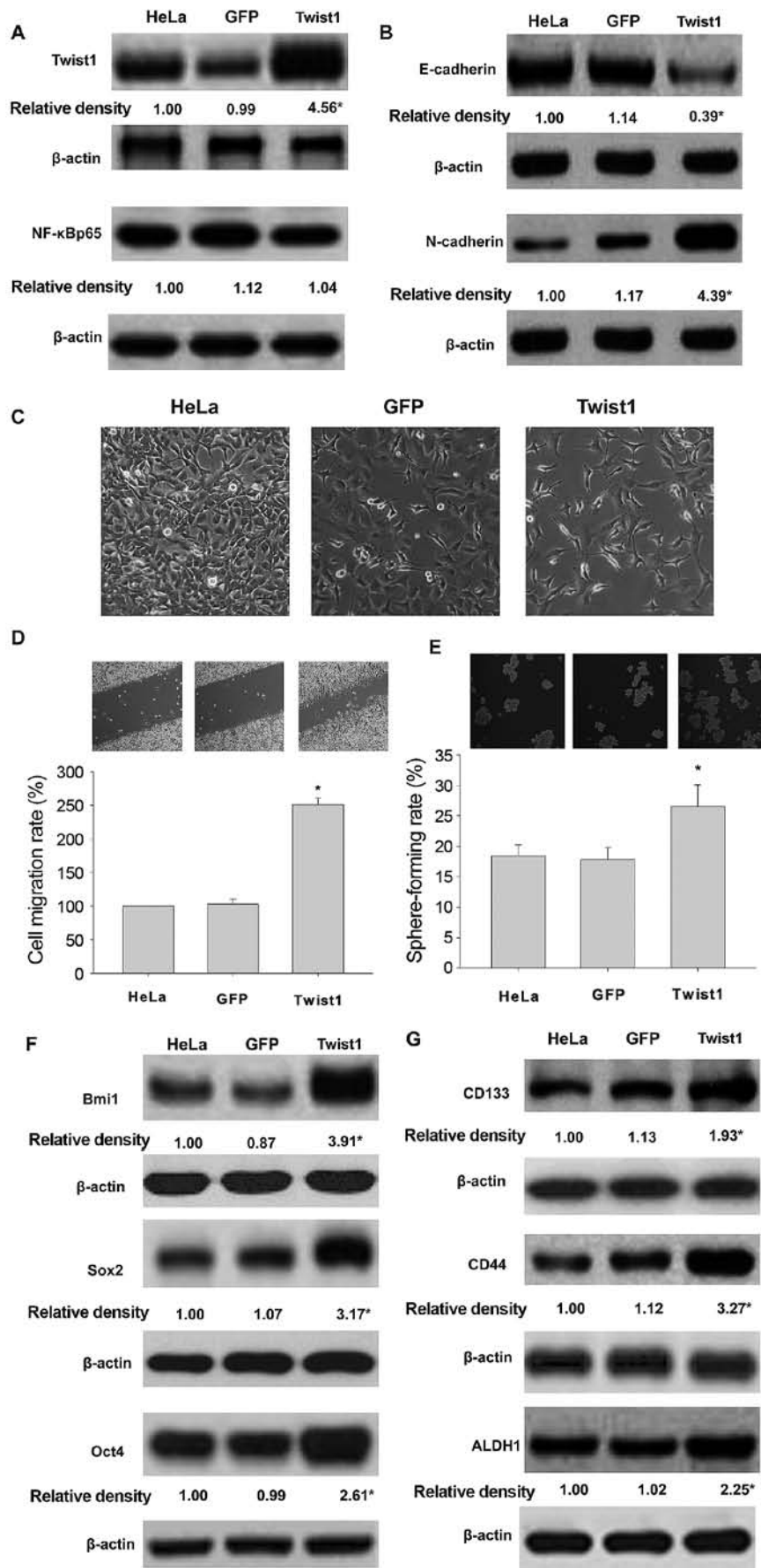


Figure 5. Overexpression of *Twist1* promotes EMT and CSCL properties in HeLa cells co-treated with TGF- β and TNF- α . Effects of *Twist1* gene transduction on the expression of Twist1 and NF- κ Bp65 (A), EMT morphology (B), E-cadherin and N-cadherin profile (C) cell migration (D), sphere formation (E), expression of Bmi1, Sox2 and Oct4 (F) and CD133, CD44 and ALDH1 (G) in HeLa cells exposed to TNF- α and TGF- β . HeLa, HeLa cells exposed to TNF- α and TGF- β . GFP, the cells transduced with *GFP* gene. Twist1, cells transduced with *Twist1* gene (* P <0.05). * P <0.05, vs HeLa or GFP.

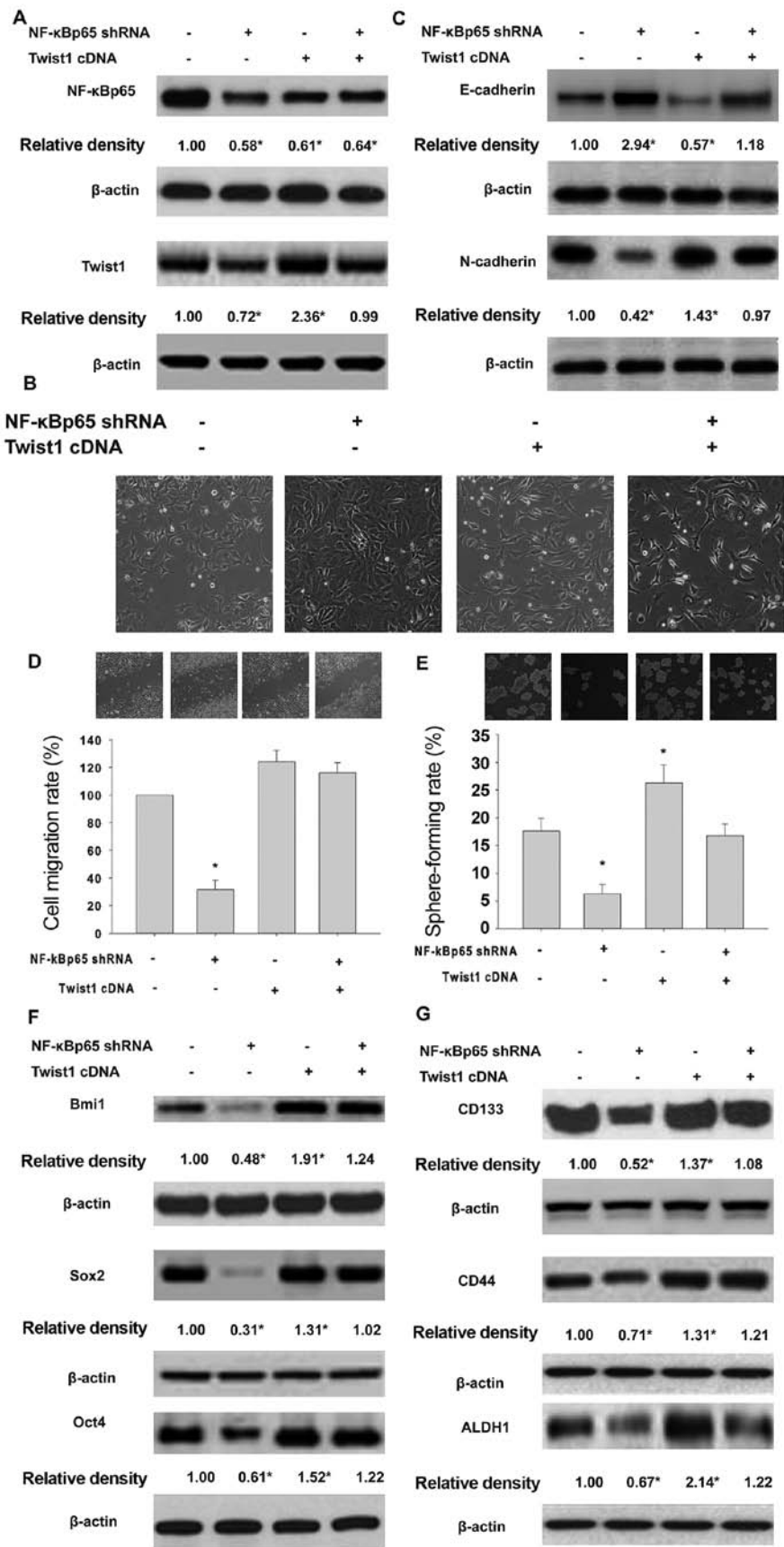


Figure 6. Effects of *Twist1* transduction in HeLa cells expressing shNF- κ Bp65 on EMT and CSCL properties following exposure to TNF- α and TGF- β . Effects of *Twist1* gene transduction in HeLa cells expressing shNF- κ Bp65 on the expression of Twist1 and NF- κ Bp65 proteins (A), EMT morphology (B), E-cadherin and N-cadherin profile (C), cell migration (D), sphere formation (E), expression of Bmi1, Sox2 and Oct4 (F), and the expression of CD133, CD44 and ALDH1 (G) induced by exposure to proinflammatory cytokines. HeLa, HeLa cells exposed to TNF- α and TGF- β . NF- κ Bp65 shRNA, shNF- κ Bp65 transduction of HeLa cells exposed to TNF- α and TGF- β . Twist1 cDNA, *Twist1* gene transduction of HeLa cells expressing NF- κ Bp65 shRNA induced by TNF- α and TGF- β . *P<0.05 vs. HeLa or NF- κ Bp65 shRNA.

Knockdown of Twist1 has no effect on NF- κ B expression but reverses the EMT and CSCL properties in HeLa cells. To elucidate the relationship between NF- κ B and Twist1 in HeLa cells, shTwist1-expressing adenovirus was used to infect HeLa cells following exposure to TNF- α and TGF- β . Compared with GFP-expressing HeLa cells, the knockdown of Twist1 downregulated the expression of Twist1 but not the expression of NF- κ Bp65 (Fig. 4A). Twist1 knockdown resulted in cobble-stone-like cells (Fig. 4B). The expression of E-cadherin was significantly upregulated while the mesenchymal marker N-cadherin was downregulated (Fig. 4C). Further, Twist1 silencing attenuated the migration and self-renewal of HeLa cells exposed to TNF- α and TGF- β (Fig. 4D and E). Furthermore, Twist1 knockdown downregulated cancer stem-related multi-functional proteins Bmi1, Sox2 and Oct4 (Fig. 4F) and surface markers CD133, CD44 and ALDH1 in HeLa cells following co-treatment with TNF- α and TGF- β (Fig. 4G).

Overexpression of Twist1 promoted EMT and CSCL properties of HeLa cells exposed to TNF- α and TGF- β . Overexpression of Twist1 was not accompanied by NF- κ Bp65 protein expression (Fig. 5A). As shown in Fig. 5B and C, Twist1-expressing adenovirus infected HeLa cells overexpressing mesenchymal marker N-cadherin and downregulated the epithelial marker E-cadherin. Furthermore, overexpression of Twist1 increased cell migration (Fig. 5D) and high sphere formation rate (Fig. 5E). Twist1 regulated the expression of Bmi1, Sox2, and Oct4 (Fig. 5F), and CD133, CD44 and ALDH1 (Fig. 5G) in HeLa cells following co-treatment with TNF- α and TGF- β .

Twist1 transduction rescues NF- κ B knockdown-related inhibition of Twist1 expression, and EMT and CSCL properties in HeLa cells exposed to inflammatory cytokines. To elucidate the role of NF- κ B/Twist axis in EMT and CSCL properties of HeLa cells exposed to inflammatory cytokines, we transduced *Twist1* into NF- κ Bp65-silenced HeLa cells. As shown in Fig. 6A, NF- κ Bp65 shRNA downregulated NF- κ Bp65 and Twist protein expression. Transduction of *Twist1* upregulated the levels of Twist without affecting the NF- κ Bp65 profile. Interestingly, *Twist1* gene transduction restored NF- κ Bp65 shRNA-downregulated Twist expression. The shNF- κ Bp65-expressing HeLa cells overexpressed epithelial markers E-cadherin and downregulated mesenchymal marker N-cadherin. Under these conditions, *Twist1* gene transduction did not inhibit NF- κ Bp65, but rescued shNF- κ Bp65 down-regulation of Twist 1 expression (Fig. 6B and C). Similarly, *Twist1* gene transduction rescued the inhibitory effects of shNF- κ Bp65 on migration (Fig. 6E) and self-renewal (Fig. 6F) and cancer stem cell-related protein expression (Fig. 6G) in HeLa cells following exposure to TNF- α and TGF- β .

Discussion

Chronic inflammation-induced carcinogenesis and metastasis is a major challenge to cancer therapy, and is a key factor contributing to mortality in many malignancies (11-17). Understanding the mechanisms regulating the metastasis and carcinogenesis induced by pro-inflammatory cytokines may lead to novel therapeutic interventions (17). In this study, we demonstrated that pro-inflammatory TNF- α and TGF- β synergistically induced

EMT and CSCL properties in HeLa cells via NF- κ B/Twist axis. We characterized the biological role of NF- κ B and Twist1 in EMT and cell migration, self-renewal and stem cell marker expression. We demonstrated the role of NF- κ B/Twist1 signal axis in HeLa cells induced by exposure to TNF- α and TGF- β . Various studies suggest that TNF- α induces a variety of epithelial cells and epithelial tumor cell EMT morphology following chronic exposure to TGF- β (17). In this study, we constructed a chronic inflammation model, by co-treatment with TNF- α and TGF- β to induce EMT phenotype in HeLa cells. Further, the exposure to pro-inflammatory cytokines also leads to cell migration and self-renewal, and CSC-related protein expression. NF- κ Bp65 knockdown or overexpression therefore, alters Twist1 protein expression. However, Twist1 knockdown or overexpression has no effects on NF- κ Bp65 expression. The results provide convinced evidence supporting NF- κ B location upstream of Twist1. Finally, we demonstrated the role of NF- κ B/Twist axis, using *Twist* and *shNF- κ Bp65* co-transduction rescue assay. The results show that Twist1 overexpression almost reversed all the biological effects of shNF- κ Bp65.

An increasing number of studies have shown that EMT plays a decisive role in tumorigenesis, including local infiltration and metastasis and spread through the circulatory system (26). We monitored the cell morphology and the expression of EMT-related proteins E-cadherin and N-cadherin to determine the phenotype variation. E-cadherin triggers epithelial inter-cellular adhesion. Cells devoid of E-cadherin show increased N-cadherin expression (27). In this study, the role of NF- κ B/Twist signal axis in EMT phenotype acquisition by HeLa cells was examined, and their overexpression promoted EMT.

Migration and CSL properties increase the risk of malignant tumor metastasis (28-30). Therefore, we investigated these phenomena along the NF- κ B/Twist axis, using scratch assay and sphere formation to detect migration and self-renewal. NF- κ B/Twist overexpression promotes HeLa cell migration and self renewal. Similarly, NF- κ B/Twist overexpression upregulates the levels of CSC proteins Bmi1, Sox2 and Oct4 and CSC surface proteins CD133, CD44, and ALDH1.

In conclusion, our results provide insight into the mechanism of TNF- α -induced EMT and CSCL properties of HeLa cells chronically exposed to TGF- β , and demonstrate that these effects are mediated via NF- κ B/Twist axis. Targeting NF- κ B/Twist axis is a potential treatment strategy to improve prognosis in patients with cervical cancer.

Acknowledgements

This study was supported by the Projects of NSFC (nos. 30760248, 81172375 and 31400311), the Project of Scientific Research Fund of Hunan Provincial Education Department (no. 14C0707), the Project of Hunan Provincial Natural Science Foundation (no. 13JJ3061) and the Scientific Research Fund of Hunan Normal University (nos. 140668 and 140666).

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