Original Paper

Chrysin Inhibits Proinflammatory Factor-Induced EMT Phenotype and Cancer Stem Cell-Like Features in HeLa Cells by Blocking the NF-κB/Twist Axis

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Abstract
Background/Aims: TNF-α and TGF-β associated epithelial-mesenchymal transition (EMT) occurs via NF-κB-dependent transcriptional upregulation of Twist1.Chrysin (ChR) is a major active ingredient ofpropolis, which inhibits various cancer cells and possesses anti-inflammatory activities. This study aimed to assess whether and how ChR inhibits proinflammatory cytokine-induced EMT phenotype and cancer stem-like cell (CSLC) features in the HeLa cell line. ***Methods:*** HeLa cells were co-administered TNF-α (10.0 ng/mL) and TGF-β (5.0 ng/mL) for 24h following TGF-β (5.0 ng/mL) alone for 6 d in the presence or absence of ChR (5.0, 10.0 and 20.0μM). Then, the levels of EMT-related factors, multi-potential transcription factors, and stem cell markers were analyzed by immunoblot. Wound healing and tumor sphere formation assays were performed to assess the migration and self-renewal capabilities of cells, respectively. Overexpression and/or knockdown of NF-κBp65 and/or Twsit1 were used to explore the molecular mechanisms. ***Results:*** The results showed that ChR inhibited EMT and CSLC properties in HeLa cells administered TNF-α after prolonged TGF-β treatment, in a concentration-dependent fashion. NF-κBp65 knockdown and ChR(10.0μM) cooperatively enhanced the inhibition of NF-κBp65 and Twist1 expression, EMT, and CSLC properties. Conversely, overexpression of NF-κBp65 combined with ChR(10.0μM) antagonized such activities. Meanwhile, Twist1silencing or overexpression combined with ChR treatment did not affect NF-κBp65 levels, but also reduced or enhanced EMT and CSLC properties. Importantly,

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overexpressing Twist1 combined with ChR reversed the effects of NF-κBp65 knockdown and ChR. ***Conclusion:*** ChR inhibits proinflammatory cytokine-induced EMT and CSLC features in HeLa cells by blocking the NF-kB/Twist axis.

Introduction

Cervical carcinoma represents one of the most frequently diagnosed malignancies, and the number 1 killer among cancers affecting the female population in underdeveloped regionsglobally [1, 2]. It can be caused by chronic infection with high-risk human papillomavirus (HPV) [3-5]. Besides, cytokine expression patterns change after malignant transformation, and inherited polymorphisms of cytokine genes might affect the risk of cervical carcinoma [6, 7]. Elevated cytokines in chronic inflammation of the cervix are closely related to cervical cancer. However, metastasis is the primary cause of treatment failure and results in the death of patients with cervical cancer [8]. Therefore, further exploration of new therapeutic agents is necessary to improve therapeutic outcomes in human cervical cancer.

In recent years, cancer stem cells (CSCs) are considered to have self-renewal ability; in addition, they can differentiate into multiple cell lines and cause cancer [9, 10]. The existence of CSCs is increasingly more convincing. Tumors, including cervical carcinoma, comprise CSCs with both epithelial and mesenchymal properties, as well as epithelialcells [11, 12]. Several studies have found that epithelial-mesenchymal transition (EMT) is the transdifferentiation of epithelial cells into motile mesenchymal cells, and contributes to tumor progression, invasion, metastasis, and dissemination [13-15]. After epithelial cells undergo EMT, they show stem-like properties [16-18]. Co-treatment with TGF-β and TNF-α promotes EMT processes, which contributes to breast CSC phenotypic characteristics [19, 20]. Therefore, inhibiting the EMT phenotype and CSC characteristics is a promising therapeutic strategy.

NF-κB signaling is tightly involved in cytokine production and immune responses, which participate in the maintenance of a variety of stem cells [21]. Twist1, a master regulator of morphogenesis, modulates EMT in multiple malignancies, e.g. prostate, uterus, and breast carcinomas [22-24]. Studies have demonstrated that TNF-α promotes EMT through AKT/GSK or NF-κB-dependent Snail and Twist expression in several cancers [25-27]. We recently reported that TNF-α and TGF-β jointly induce carcinogenesis through NF-κB/Twist signaling [28]. Therefore, the NF-κB/ Twist axis is strongly associated with EMT and CSCs, which may provide vital therapeutic targets for preventing human cervical cancer.

Chrysin(ChR), a natural flavonoid, is usually found in honey, bee propolis, and plants [29]. Previous studies have shown that ChR exerts strong anti-inflammatory, antioxidative, and anticancer effects [30, 31]. In addition, ChR promotes apoptosis in the cervical cancer Hela cells by different mechanisms [32, 33]. However, it remains unclear whether and how ChR suppresses the EMT phenotype and CSC properties of HeLa cells induced by proinflammatory factors.

Therefore, the current study aimed to assess whether ChR inhibits the EMT phenotype and tumor stem cell-like properties of HeLa cells induced by proinflammatory factors via the NF-kB/Twist signaling axis, thereby providing critical therapeutic targets for intervention in human cervical cancer.

Materials and Methods

Reagents

ChR was obtained from Sigma-Aldrich (USA). TGF-β and TNF-α were produced by Sino Biological (China). Anti-β-actin antibody was obtained from Santa Cruz Biotechnology (USA). Anti-Lamin B antibody was obtained from SHANGHAI ANYAN TRADE CO., LTD (Shanghai, China). Primary antibodies raised against E-cadherin and N-cadherin were obtained from Cell Signaling Technology (Danvers, MA, USA); antibodies targeting Bmi1, Sox2, Oct4, CD133, CD44, ALDH1, NF-κB and Twist1 were obtained from Abcam

(USA). Anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were manufactured by Beyotime Institute of Biotechnology (China). The chemiluminescent substrate was produced by Amersham Pharmacia Biotech (Piscataway, NJ, USA). Enhanced infection solution was from GeneChem (ENi.s, Catalog No. REVG0002; China). Opti-MEM was supplied by Invitrogen (China). Adenoviral particles packaged with control pHBad-U6-GFP, pHBAd-MCMV-GFP, pHBad-U6-GFP-sh NF-κBp65, pHBAd-MCMV-GFP-NF-κBp65, pHBad-U6-GFP-shTwist, and pHBAd-MCMV-GFP-Twist plasmids, respectively, were obtained from Hanbio Biotechnology Co. Ltd. (1.0 mL, 1×10^{11} PFU/ mL; China).

Cell culture and Treatment

HeLa cells obtained from the Cell Bank of Chinese Academy of Sciences (China), were maintained according to previously described methods [34]. For modeling, the cells were treated with TNF-α (10.0 ng/mL) for 24h, TGF-β (5.0 ng/mL) for 6d, or TNF-α (10.0 ng/mL) combined with TGF-β (5.0 ng/mL) for 24h following TGF-β (5.0 ng/mL) alone administered for 6d. For drug intervention, cells were administered ChR at different amounts (5.0, 10.0 and 20.0μM) for 24hor ChR(10.0μM) for 0h, 0.5h, 3h, 6h, 12h and 24h, respectively. To explore mechanisms, HeLa cells expressing NF-κBp65 shRNA or NF-κBp65, Twist1 shRNA or Twist1 were treated with or without ChR (10.0μM) for 24h. EMT features were assessed under a light microscope (Olympus, Japan).

Cellular Fractionation Analysis

Cellular fractionation was performed as described by Li J. et al. [34]. Briefly, cells were harvested with trypsinization and washed twice with phosphate-buffered saline (137 mM NaC1, 2.7 mM KC1, 4.3 mM Na$_2$HPO$_4$, 1.4mM KH$_2$PO$_4$, pH 7.4). Cells were rapidly washed once with hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 10 mMKCl, 0.2 mM PMSF, and 0.5 mM DTT), re-suspended with 3 packed cell volume of hypotonic buffer and allowed to swell on ice for 10 min. Cells were then homogenized with 20 strokes on Dounce homogenizer (type B pestle) to ensure that >95% of cells were lyzed. After centrifugation at 4°C with 3300 × g for 15 min, supernatant was saved for S-100 cytoplasmic extract preparation. The nuclear pellet was washed once with lysis buffer (50 mMTris-HCl pH7.4, 150 mMNaCl, 0.2 mM EDTA, 0.2% NP-40, 10% Glycerin, and protease cocktail) and suspected in the same buffer. After brief sonication, the suspension was spin at 13, 200 × g for 20 min and supernatant was saved as the nuclear fraction.

Wound-healing assay

HeLa cells were cultured until optimal confluence (80–90%). Then, the monolayer was gently and slowly scratched with a new 100μL pipette tip across the center of the well. After treatment for 0h and 24h, respectively, the cells were washed twice with PBS, and imaged in the same field for analysis; mock treatment was used for standardizing the number of migrated cells.

Sphere formation assay

Single-cells were seeded into ultra-low attachment 24-well plates (Corning, USA) at 10^3 cells/well in serum-free stem cell conditional medium. After a 5 day incubation period, spheroids were counted, to derive the sphere-formation rate by dividing the total number of spheres obtained by that of live cells seeded multiplied by one-hundred.

Immunoblot

Immunoblot was carried out according to a previous report [35]. The primary antibodies used for membrane incubation targeted E-cadherin, N-cadherin, Bmi1, Sox2, Oct4, CD133, CD44, ALDH1, NF-κBp65 and Twist. The membranes were further incubated with appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were revealed by enhanced chemiluminescence. The UN-SCAN-IT software (Silk Scientific) was used for densitometric analysis, with β-actin serving as an internal reference.

NF-κB binding activity assay

The NF-κB activities in the nuclear protein (20 μg) of HeLa cells treated with TNF-α (10.0 ng/mL) combined with TGF-β (5.0 ng/mL) for 0h, 0.5h, 3h, 6h, 12h and 24h in the presence or absence of ChR (10.0 μM) following TGF-β (5.0 ng/mL) alone administered for 6d were measured using a DNA-binding ELISA kit

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(TransAM™ NF-κBp65 assay; Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions, respectively. Absorbance at 405 nm with an enzyme-labeling instrument (ELX-800 type; Bio-Tek, Shanghai, China) was represented as NF-κB activities.

Cell transduction

Cells grown in 24-well culture plates to 40-50% confluence were incubated overnight. Subsequently, they were infected with adenoviral particles packaged with control pHBad-U6-GFP or pHBAd-MCMV-GFP and pHBad-U6-GFP-shNF-κBp65, pHBAd-MCMV-GFP-NF-κBp65, pHBad-U6-GFP-shTwist1, and pHBAd-MCMV-GFP-Twist1 plasmids, respectively, cultured with Opti-MEM using the enhanced infection solution (ENi.s; GeneChem, China). Following 4h of transduction, DMEM containing 10% FBS was added to replace the transduction medium. The cells were then incubated for an additional 48h, prior to protein level assessments.

In vivo tumorigenicity assay

The animal experiments were carried out according to standard protocols, after approval from the Ethics Committee of Hunan Normal University and the Committee of Experimental Animal Feeding and Management. Hela cells exposed to TNF-α(10.0 ng/mL) combined with TGF-β(5.0 ng/mL) for 24h following TGF-β (5.0 ng/mL) treatment for 6d and untreated cells were diluted to 1.0×10^6 cells/50μl/mouse, added to Matrigelin equal proportions, and subcutaneously injected to four four-week-old female BALB/c-nu mice (12-14 g). After one month, the mice were sacrificed under deep anesthesia with pentobarbital. Tissue samples from experimental and control animals were fixed with 10% neutral-buffered formalin for 24h, and paraffin embedded. Finally, sections (5μm) were prepared for H&E staining.
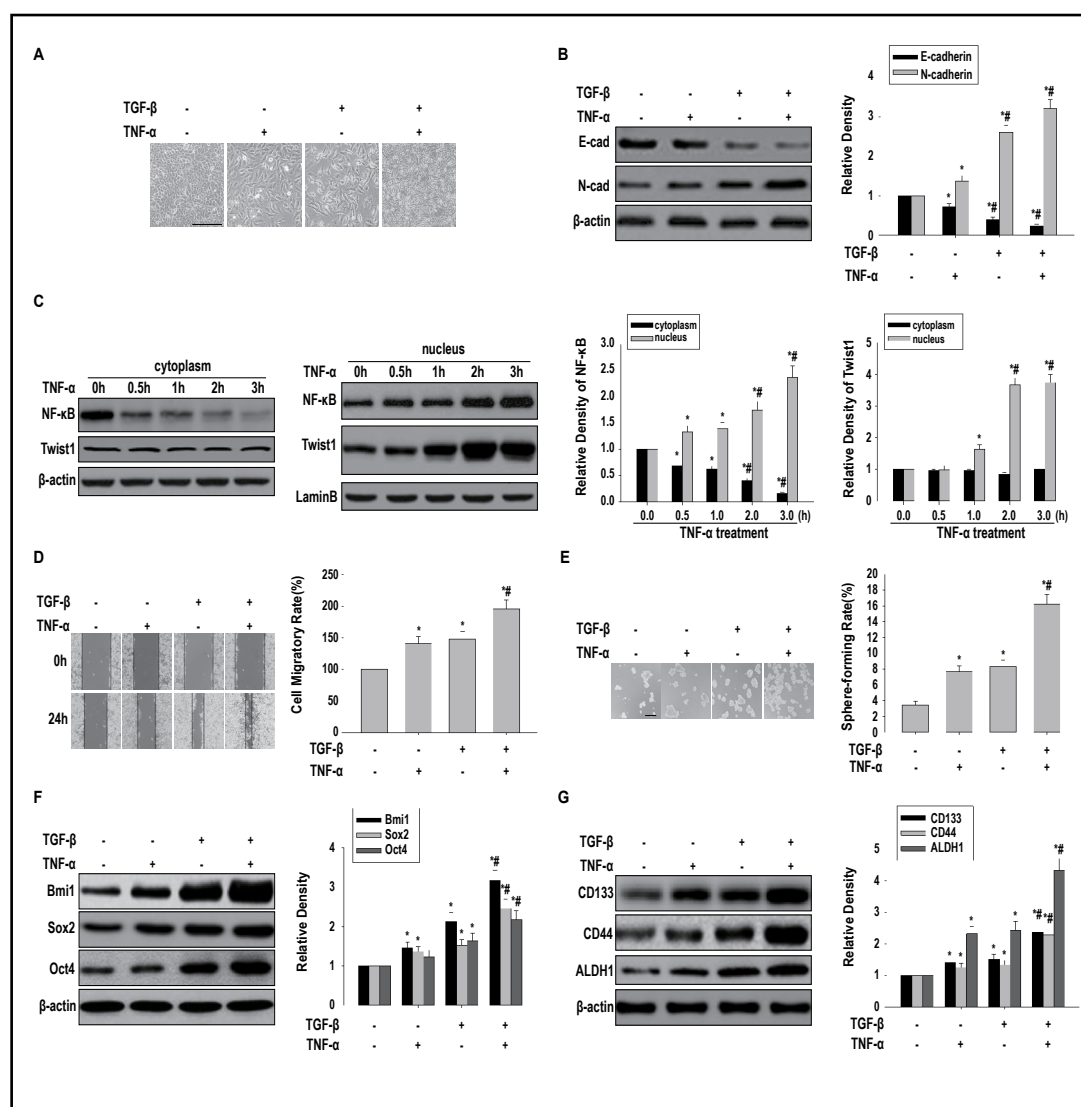
Statistical analysis

At least three independent experiments were performed, and data are mean±standard deviation (SD), and analyzed by one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ indicated statistical significance.

Results

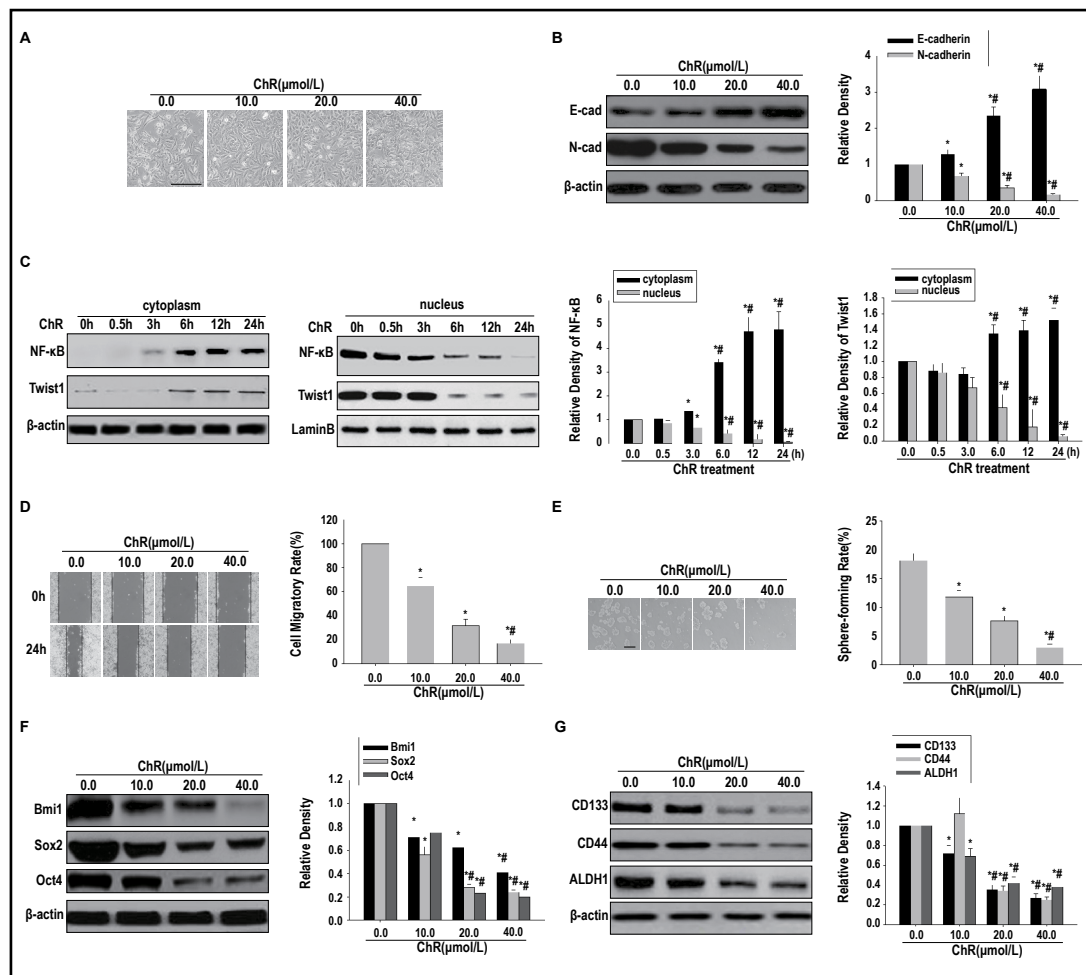### Co-treatment with TNF-α and TGF-β promotes EMT and CSLC features in HeLa cells

We previously reported that HeLa cells administered TNF-α after prolonged induction by TGF-β showed EMT and CSCLC features through NF-κB activation and Twist1 upregulation [28]. To further confirm that TNF-α and/or TGF-β promote EMT and CSCLC features, HeLa cells were exposed to TNF-α and/or TGF-β. As expected, TNF-α and TGF-β alone or in combination induced EMT, reflected by morphological alterations from a cobble-stone-like shape to a spindle-like phenotype, especially in cells administered both TNF-α and TGF-β (Fig. 1A). Fig. 1B show that TGF-β and/or TNF-α resulted in E-cadherin downregulation, and N-cadherin upregulation, as demonstrated by Western blot analysis. Given TNF-α activation induces NF-κBp65 nuclear translocation, we examined endogenous NF-κBp65 and Twist1 localization in HeLa cells and found that TNF-α following prolonged induction by TGF-β induced nuclear translocation of NF-κBp65 and Twist1 at 0.5 h and 1h post treatment respectively, suggesting TNF-α triggers a dynamic interaction between nuclear translocation of NF-κBp65 and nuclear expression of Twist1(Fig. 1C). Our results also demonstrated that proinflammatory cytokines jointly increased the migration (Fig. 1D) and self-renewal (Fig. 1E) capabilities of HeLa cells. Meanwhile, the multipotency factors Bmi1, Sox2, and Oct4 (Fig. 1F), and stem cell markers CD133, CD44, and ALDH1 (Fig. 1G) were up-regulated. Importantly, Hela cells administered TNF-α after prolonged treatment with TGF-β showed enhanced xenograft growth, as reflected by tumor size and weight (Supplementary Fig. 1 – for all supplemental material see www.cellphysiolbiochem.com). These results confirmed that HeLa cells co-treated with TNF-α and TGF-β showed EMT and CSLC characteristics.

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Fig. 1. Co-administration of TGF-β and TNF-α contributes to EMT and CSLC features in HeLa cells. HeLa cells co-administered TNF-α and TGF-β displayed mesenchymal morphology(A, scale bar, 50 μm), reduced E-cadherin and elevated N-cadherin levels(B), increasednuclear translocation of NF-κBp65 and Twist1(C), enhanced migration (D) and self-renewal (E, scale bar, 200 μm) abilities, and upregulated Bmi1, Sox2 and Oct4 (F)as well as CD133, ALDH1 and CD44(H).*$p<0.05$, vs untreated cells;#$p<0.05$, vs treated with TNF-α or TGF-β. (C) HeLa cells were treated with TNF-α followed by prolonged induction by TGF-β at different time point. Cytosolic and nuclear NF-κBp65 and Twist1 protein were separated using hypotonic buffer. β-actin and lamin B indicate cytosolic and nuclear fraction, respectively.

ChR inhibits EMT and CSLC features in HeLa cells induced by TNF-α and TGF-β co-administration

To assess the effect of chrysin on EMT and CSLC features in HeLa cells induced by proinflammatory cytokines, HeLa cells were treated with TNF-α and TGF-β in combination for 24h following TGF-β administration for 6d in the presence or absence of different concentrations (5.0, 10.0, and 20.0μmol/L) of ChR. As shown in Fig. 2A, ChR could reverse the morphological changes of EMT induced by pro-inflammatory cytokines. In addition, ChR upregulated E-cadherin and downregulated N-cadherin at the protein level, in a concentration-dependent manner (Fig. 2B). To further confirm the inhibition of NF-κBp65 and Twist1 nuclear translocation by ChR, nuclear and cytoplasmic fractions of HeLa cells

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Fig. 2. Effect of ChR on EMT and CSLC features in HeLa cells induced by TNF-α combined with TGF-β. HeLa cells were co-treated with TNF-α and TGF-β with or without different concentrations (5.0, 10.0 and 20.0μM) of ChR. ChR reversed mesenchymal morphology (A, scale bar, 50 μm), regulated E-cadherin and N-cadherin (B), reduced nuclear translocation of NF-κBp65 and Twist1(C), inhibited cell migration (D) and self-renewal (E, scale bar, 200 μm) abilities, and downregulated Bmi1, Sox2 and Oct4 (F) as well as CD133, CD44 and ALDH1 (G) at the protein level.$^*p<0.05$, vs 0.1%DMSO treatment; $^#p<0.05$, vs ChR (10μM) treatment. (C) HeLa cells co-treated with TNF-α and TGF-β were treated with ChR (10.0 μM) for the indicated times. Cytosolic and nuclear NF-κBp65 and Twist1 protein were separated using hypotonic buffer. β-actin and lamin B indicate cytosolic and nuclear fraction, respectively.

were isolated at different time points upon treatment with ChR. Fig. 2C indicates that chrysin inhibited nuclear translocation of NF-κBp65 at 3h, whereas the nuclear expression of Twist1 began to reduce at 6h after treatment, suggesting ChR inhibit Twist1 nuclear expression probably by suppression of NF-κBp65 nuclear translocation. The wound-healing and sphere-forming assays revealed that ChR suppressed the migration and self-renewal abilities of HeLa cells co-treated with TNF-α during prolonged administration of TGF-β, in a dose-dependent fashion (Fig. 2D and 2E). Furthermore, compared with untreated cells, ChR significantly decreased the levels of Bmi1, Sox2 and Oct4 (Fig. 2F), as well as CD133, CD44 and ALDH1 (Fig. 2G) after co-administration of TNF-α during prolonged treatment with TGF-β. To corroborate the time procedure of inhibitory effects of ChR on NF-κB activation, we employed a DNA-binding ELISA kit to measure NF-κB activities. The results showed that the NF-κB activities elevated at 0.5 h and continued to 24 h；the inhibitory effects of ChR (10.0 μM) originated at 3h and continued 24h under the combination of TNF-α and TGF-β

treatment (Supplementary Fig. 2). These results suggest that ChR inhibited EMT and CSLC features in HeLa cells induced by co-administration of TNF-α and TGF-β may be dependent on downregulating Twist1 expression by inhibition of NF-κB activities.

NF-κBp65 knockdown cooperatively increases the inhibitory effects of ChR on EMT and CSLC features in HeLa cells induced byTNF-α and TGF-β co-administration

To assess whether inhibitory effects of ChR on EMT and CSLC features in cells induced by proinflammatory cytokines are dependent on NF-κBp65, HeLa cells expressing GFP or shNF-κBp65(Supplementary Fig. 3) were administered TGF-β and TNF-α in combination, with or without ChR (10.0µM). Fig. 3A shows that knockdown of NF-κBp65 and ChR (10.0µM) cooperatively reversed the morphological changes of EMT induced by proinflammatory cytokines. In addition, knockdown of NF-κBp65 and ChR (10.0µM) cooperatively upregulated E-cadherin and downregulated N-cadherin, NF-κBp65 and Twist1 at the protein level (Fig. 3B and 3C). Wound-healing and sphere-formation assays revealed knockdown of NF-κBp65 and ChR (10.0µM) cooperatively decreased the migration and self-renewal abilities associated with co-administration of TNF-α during prolonged TGF-β treatment of HeLa cells (Fig. 3D and 3E). Furthermore, NF-κBp65 knockdown and ChR (10.0µM) cooperatively downregulated Bmi1, Sox2 and Oct4 (Fig. 3F), as well as CD133, CD44 and ALDH1 (Fig. 3G) induced after TNF-α co-administration during prolonged TGF-β treatment. These results suggested that ChR inhibited EMT and CSLC features in HeLa cells co-treated with TNF-α and TGF-β, likely via NF-κBp65 downregulation.

NF-κBp65 overexpression attenuates the inhibitory effects of ChR on EMT and CSLC features in HeLa cells co-treated with TNF-α and TGF-β

To further confirm that the inhibitory effects of ChR on EMT and CSLC features in HeLa cells induced by proinflammatory cytokines are dependent on NF-κBp65, HeLa cells expressing GFP or NF-κBp65 (Supplementary Fig. 4) were treated with TGF-β and TNF-α in combination, with or without ChR (10.0µM). As shown in Fig. 4A, overexpression of NF-κBp65 attenuated ChR (10.0µM) associated reversal of morphological changes of EMT induced by pro-inflammatory cytokines. Fig. 4B and 4C show that overexpression of NF-κBp65 reduced ChR(10.0µM) associated upregulation of E-cadherin protein and downregulation of N-cadherin, NF-κBp65 and Twist1 at the protein level. Meanwhile, wound-healing and sphere-formation assays demonstrated that overexpression of NF-κBp65 diminished ChR (10.0µM) related reduction of migration and self-renewal abilities enhanced after co-administration of TNF-α during prolonged treatment with TGF-β in HeLa cells (Fig. 4D and 4E). Furthermore, overexpression of NF-κBp65 attenuated ChR (10.0µM) downregulation of Bmi1, Sox2, and Oct4 (Fig. 4F), as well as CD133, CD44, and ALDH1 (Fig. 4G) enhanced by co-administration of TNF-α during prolonged treatment with TGF-β. These results indicated that ChR associated inhibition of EMT and CSLC features in HeLa cells induced by co-exposure of TNF-α and TGF-β is dependent on NF-κBp65 regulation.

Knockdown of Twist1 cooperatively enhances the inhibitory effects of ChR on EMT and CSLC features in HeLa cells induced by co-administration of TNF-α and TGF-β, but has no effects on NF-κBp65 levels

To explore whether the inhibitory effects of ChR on EMT and CSLC features in HeLa cells induced by proinflammatory cytokines are involved in NF-κBp65/Twist signaling, HeLa cells expressing GFP or Twist1 shRNA (Supplementary Fig. 5) were treated with TGF-β and TNF-α in combination, with or without ChR (10.0µM). Fig. 5A shows that Twist knockdown and ChR(10.0µM) cooperatively reversed the morphological changes of EMT induced by pro-inflammatory cytokines. As shown in Fig. 5B and 5C, Twist knockdown and ChR(10.0µM) cooperatively upregulated E-cadherin and downregulated N-cadherin and Twist1 at the protein level, but did not affect NF-κBp65 protein levels. Wound-healing and sphere-formation assays revealed that Twist1 knockdown and ChR (10.0µM) cooperatively decreased the migration and self-renewal abilities enhanced by co-administration of TNF-α
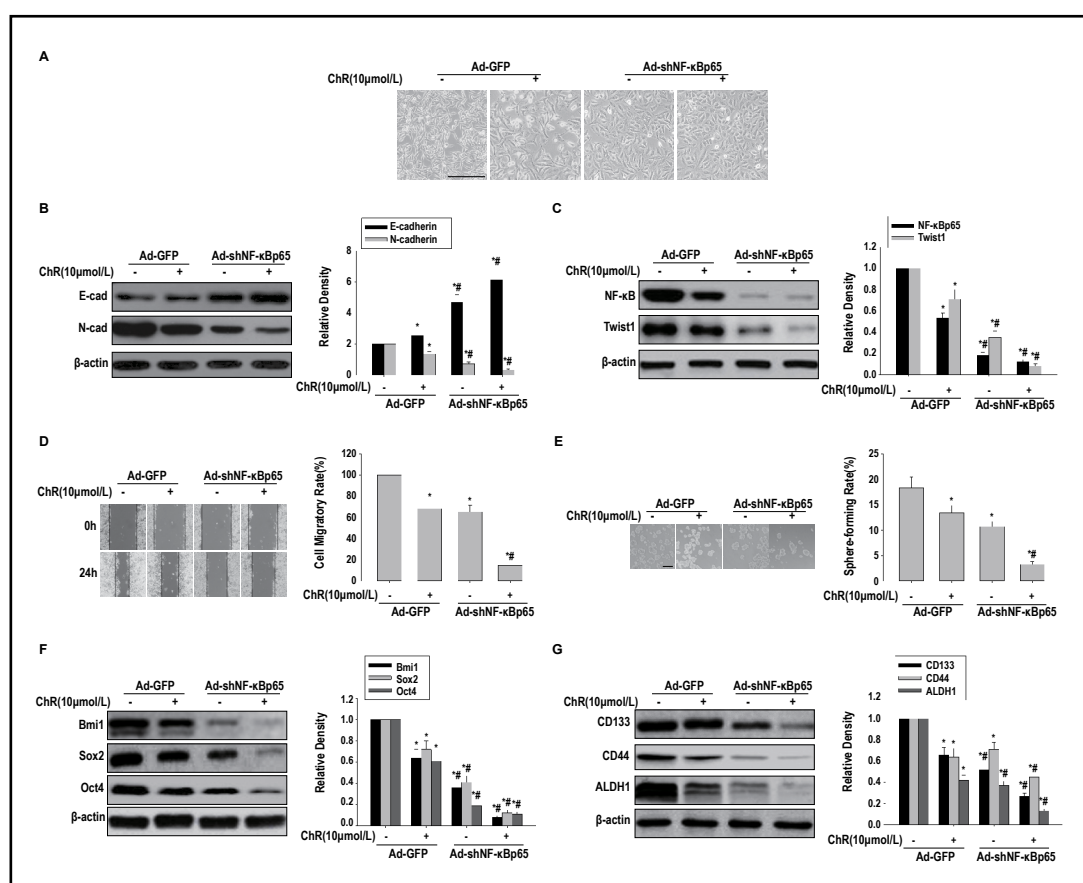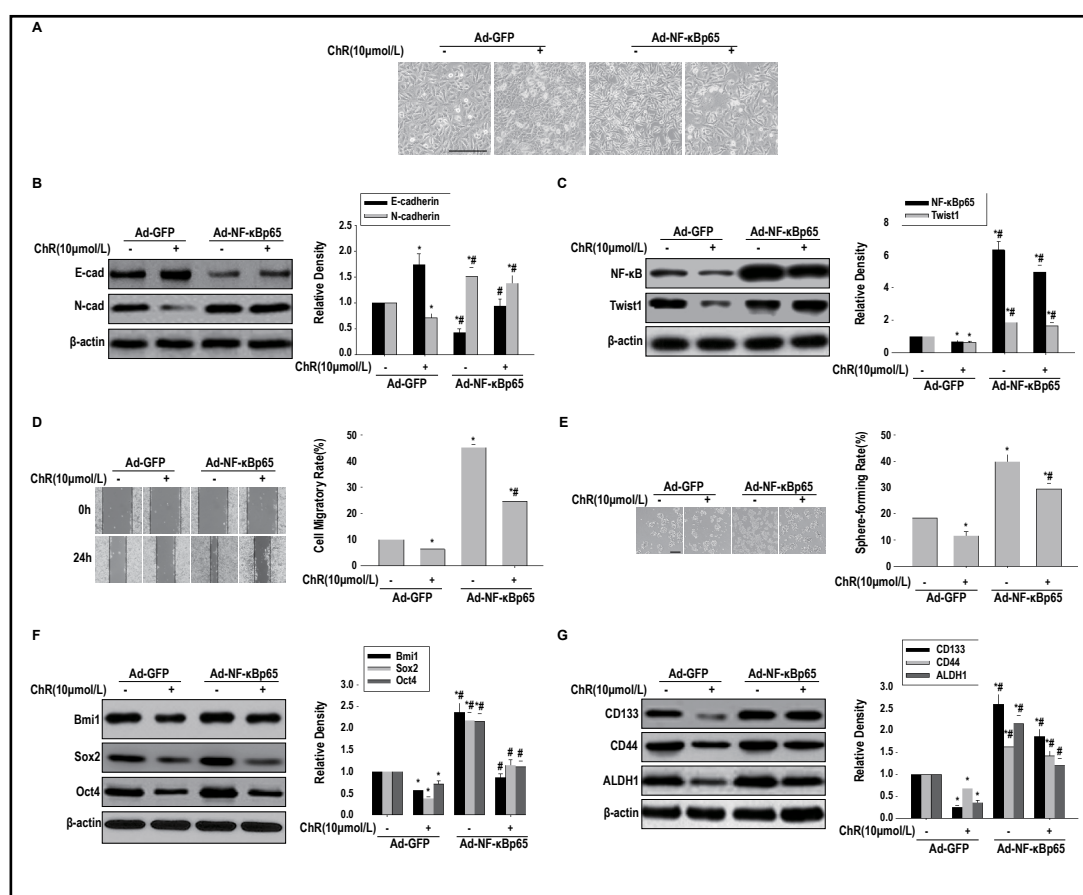
Fig. 3. Effects of NF-κBp65 knockdown on ChR associated inhibition of EMT and CSLC features in HeLa cells induced by TGF-β and TNF-α co-treatment. HeLa cells expressing shNF-κBp65 were co-administered TNF-α and TGF-β, with or without ChR(10.0μM). Then, cell morphology (A, scale bar, 50 μm), E-cadherin and N-cadherin protein levels (B), NF-κBp65 and Twist1 protein amounts (C), cell migration (D) and self-renewal (E, scale bar, 200 μm) abilities, and the protein expression levels of Bmi1, Sox2 and Oct4 (F), as well as CD133 and ALDH1 and CD44(G) were assessed. β-actin served as an internal reference for immunoblots. GFP, cells transduced with adenoviruses expressing GFP; shRNA, cells transduced with adenoviruses expressing shNF-κBp65. *p*<0.05 vs GFP;#*p*<0.05 vs GFP with ChR(10.0μM) treatment.

during prolonged treatment with TGF-β in HeLa cells (Fig. 5D and 5E). Furthermore, Twist1 knockdown and ChR (10.0μM) cooperatively the protein levels of Bmi1, Sox2 and Oct4 (Fig. 5F), as well as CD133, CD44, and ALDH1 (Fig. 5G) enhanced by co-administration of TNF-α during prolonged treatment with TGF-β. These results suggested that ChR inhibition of EMT and CSLC features in HeLa cells induced by co-exposure of TNF-α and TGF-β might be mediated by the NF-κBp65/Twist signaling axis.

Twist1 overexpression antagonized the effects of ChR on the EMT process and cancer stem cell-like properties enhanced by co-administration of TNF-α and TGF-β, but had no effects on NF-κBp65 expression

To further assess whether the inhibitory effects of ChR on EMT and CSLC features in HeLa cells induced by proinflammatory cytokines involve NF-κBp65/Twist signaling, HeLa cells expressing GFP or Twist1(Supplementary Fig. 6) were treated TGF-β and TNF-α in combination, with or without ChR (10.0μM). Fig. 6A shows that overexpression of Twist attenuated ChR (10.0μM) associated reversal of morphological changes of EMT induced by proinflammatory cytokines. As shown in Fig. 6B and 6C, overexpression of Twist1 reduced ChR(10.0μM) associated E-cadherin upregulation as well as N-cadherin and Twist1

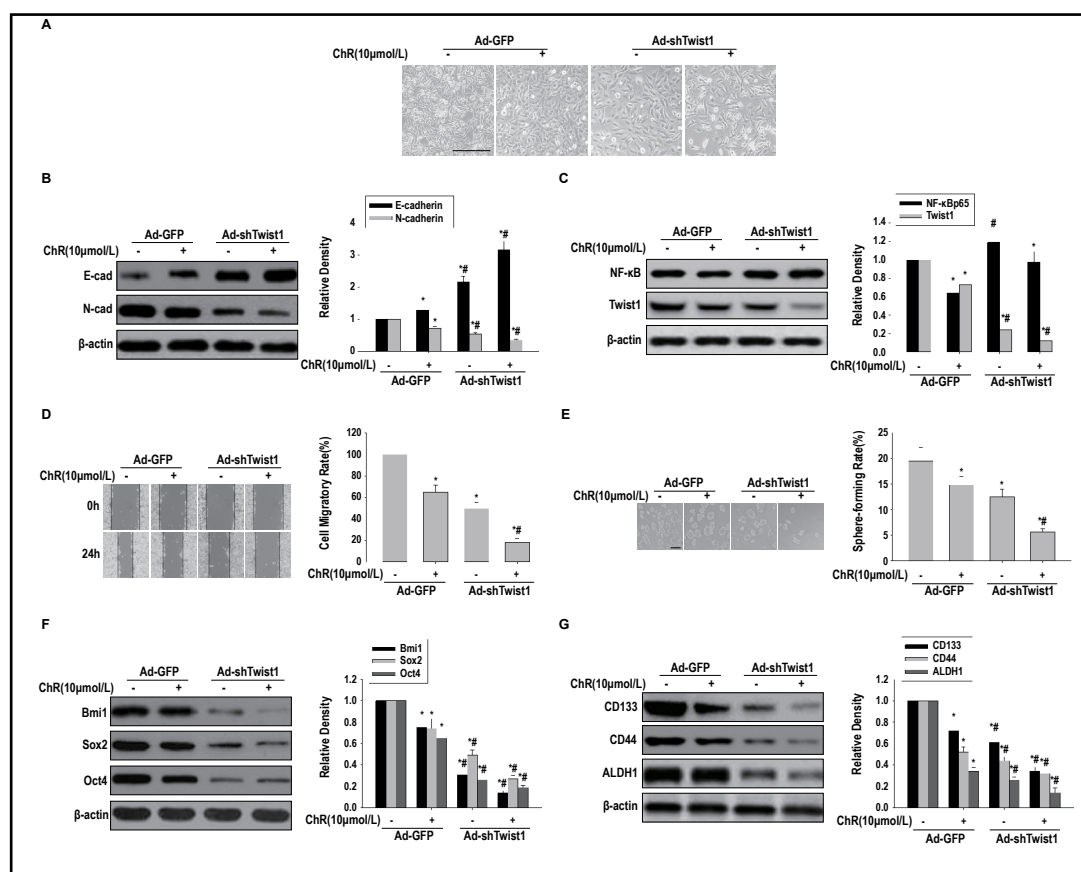Dong et al.: ChR Reverses Inflammation-StimulativeEMT in HeLa Cells

Fig. 4. Effects of NF-κBp65 overexpression on ChR associated inhibition of EMT and CSLC featuresin HeLa cells induced by TGF-β and TNF-α co-treatment. HeLa cells expressing NF-κB(p65) were co-administered TNF-α and TGF-β, with or without ChR(10.0μM). Then, cell morphology (A, scale bar, 50 μm), E-cadherin and N-cadherin protein levels (B), NF-κBp65 and Twist1 protein amounts (C), migration (D) and self-renewal (E, scale bar, 200 μm) abilities, and the protein expression levels of Bmi1, Sox2, and Oct 4(F), as well as CD133 and ALDH1 and CD44 (G) were determined. β-actin served as an internal reference for immunoblots. GFP, cells transduced with adenoviruses harboring the GFP gene; cDNA, cells transduced with adenoviruses expressing NF-κBp6.*$p<0.05$ vs GFP. #$p<0.05$ vs GFP with ChR(10.0μM) treatment.

downregulation at the protein level, but had no effects on NF-κBp65 protein levels. Wound-healing and sphere-formation assays demonstrated Twist1 overexpression diminished ChR (10.0μM) associated inhibition of migration and self-renewal abilities enhanced by co-administration of TNF-α during prolonged treatment with TGF-β in HeLa cells (Fig. 6D and 6E). Furthermore, overexpression of Twist1 attenuated ChR (10.0μM) associated downregulation of Bmi1, Sox2, and Oct4 (Fig. 6F), as well as CD133, CD44, and ALDH1 (Fig. 6G), whose levels were enhanced by co-administration of TNF-α during prolonged treatment with TGF-β. These findings indicated that ChR associated inhibition of EMT and CSLC features in HeLa cells induced by co-exposure of TNF-α and TGF-β involved the NF-κBp65/Twist signaling axis.

Overexpression of Twist1 rescues the inhibitory effects of ChR on EMT and CSLC properties induced by co-administration of TNF-α and TGF-β

To verify whether ChR inhibited EMT and CSLC features in HeLa cells induced by TNF-α combined with TGF-β through the NF-κB/Twist axis, Twist1 was overexpressed in NF-κBp65 knockdown HeLa cells. The results showed that NF-κBp65 shRNA reduced NF-κB and Twist1 protein expression levels (Fig. 7C); meanwhile, Twist1 overexpression upregulated

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Fig. 5. Effects of Twist knockdown on ChR associated inhibition of EMT and CSLC features in HeLa cells induced by TGF-β and TNF-α co-treatment. HeLa cells expressing shTwist1 were co-administered TNF-α and TGF-β, with or without ChR(10.0μM). Then, cell morphology (A, scale bar, 50 μm), E-cadherin and N-cadherin protein amounts (B), NF-κBp65 and Twist1 protein levels (C), cell migration (D) and self-renewal (E, scale bar, 200 μm) abilities, and the protein expression levels of Bmi1, Sox2, and Oct4 (F) as well as CD133, ALDH1m and CD44(G) were evaluated. β-actinserved as an internal reference for immunoblots. GFP, cells transduced with adenoviruses carrying the GFP gene; shRNA, cells transduced with adenoviruses expressing shTwist1. $^*p<0.05$ vs GFP. $^\#p<0.05$ vs GFP with ChR(10.0μM) treatment.

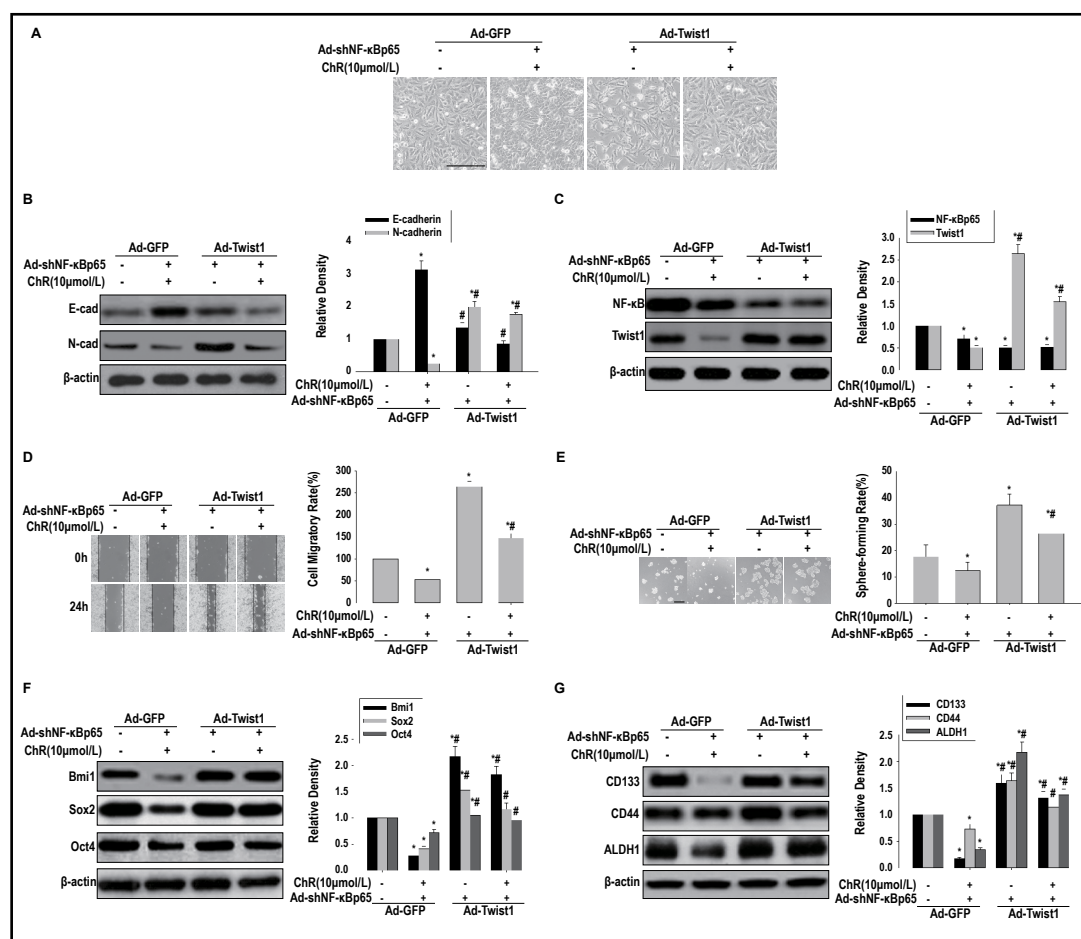Twist1 protein but did not affect NF-κB protein levels. However, Twist1 overexpression rescued NF-κBp65 shRNA associated downregulation of the Twist protein. HeLa cells by expressing shNF-κBp65 were upregulated the epithelial marker E-cadherin and down-regulated the mesenchymal marker N-cadherin in a cobble-stone-like shape. Furthermore, overexpression of Twist1 had no negative effects on NF-κBp65 expression, but could rescue shNF-κBp65 associated Twist1 downregulation (Fig. 7A and 7B). Similarly, overexpression of Twist1 could rescue the inhibitory effects of shNF-κBp65 combined with ChR (10μM) on migration(Fig. 7D) and self-renewal(Fig. 7E) abilities, as well as the expression levels of CSLC associated proteins (Fig. 7F and 7G) in HeLa cells co-treated with TNF-α during prolonged administration of TGF-β. These findings demonstrated that ChR associated inhibition of EMT and CSLC features in HeLa cells induced by TNF-α combined with TGF-β occurs via the NF-κB/Twist axis.
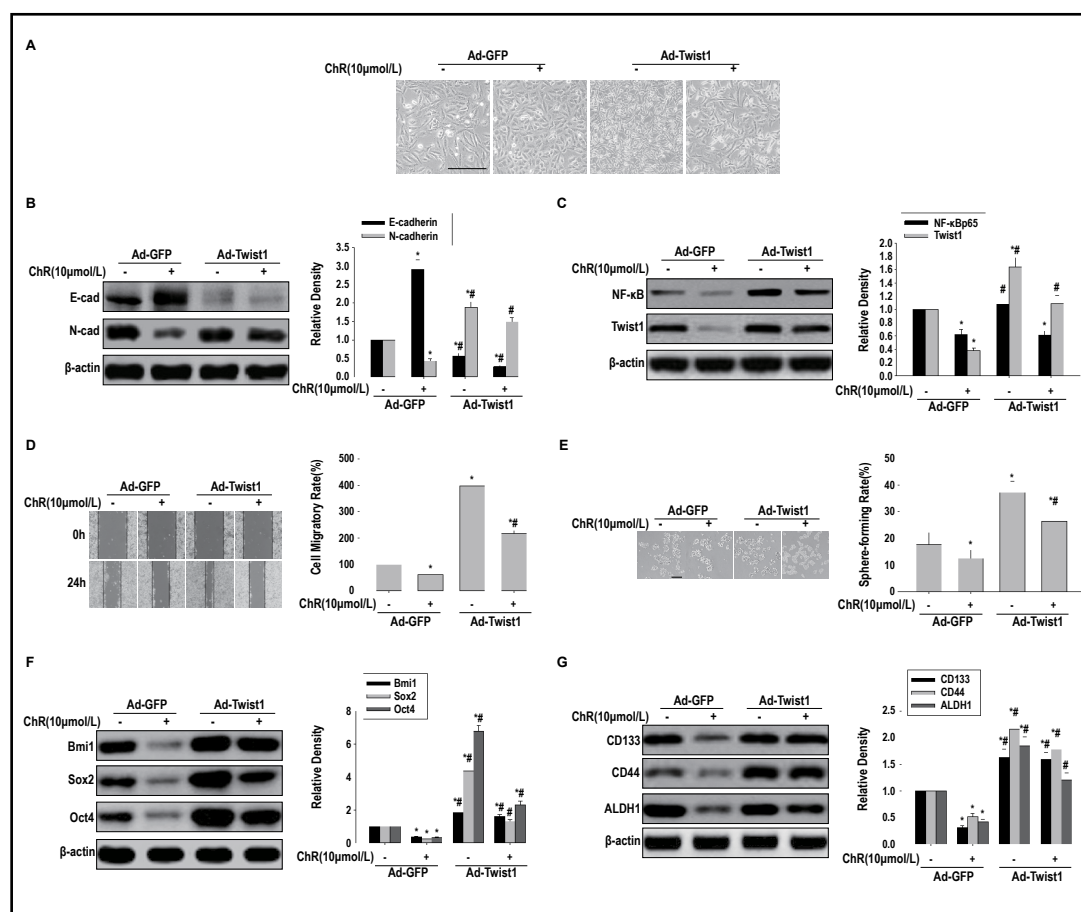
Fig. 6. Effects of Twist overexpression on ChR associated inhibition of EMT and CSLC features in HeLa cells induced by TGF-β and TNF-α co-treatment. HeLa cells expressing Twist1 were co-administered TNF-α and TGF-β, with or without ChR(10.0μM). Then, cell morphology (A, scale bar, 50 μm), E-cadherin and N-cadherin protein levels (B), NF-κBp65 and Twist1 protein amounts (C), cell migration (D) and self-renewal (E, scale bar, 200 μm) abilities, and the protein expression levels of Bmi1, Sox2, and Oct4 (F) as well as CD133, CD44, and ALDH1(G) proteins were assessed. β-actinserved as an internal reference for immunoblots. GFP, cells transduced with adenoviruses harboring the GFP gene; cDNA, cells transduced with adenoviruses expressing Twist1. $^*p<0.05$ vs GFP. $^\#p<0.05$ vs GFP with ChR(10.0μM) treatment.

Discussion

Compelling evidence indicates that chronic unabated inflammation is critical for tumor progression in many malignancies, including cervical cancer [36, 37]. Multiple studies revealed the metastatic nature and carcinogenesis of tumors induced by proinflammatory cytokines [6, 7, 38]. In the present work, a chronic inflammation model of co-administration of TNF-α with prolonged exposure to TGF-β conferred the EMT phenotype and promoted cell migration and self-renewal abilities, while upregulating CSLC associated proteins in HeLa cells. Lv N et al. found that Twist1 modulates EMT via NF-κB signaling in papillary thyroid cancer [39]. We recently reported that TNF-α in combination with TGF-β promotes EMT and CSLC properties of HeLa cells through NF-κB/Twist signaling [28]. Consistently, the present study confirmed the biological function of the NF-κB/Twist axis in EMT and CSLC properties such as migration and self-renewal abilities, and the expression levels of CSLC markers in HeLa cells co-treated with TNF-α during prolonged administration of TGF-β. We further found that the proinflammatory factors TNF-α and TGF-β in the tumor microenvironment activated the NF-κB/Twist axis to induce EMT and CSLC properties in HeLa cells as shown

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Fig. 7. Twist1 overexpression reverses the inhibitory effects of shNF-κB combined with ChR on EMT and CSLC features in HeLa cells induced by TNF-α and TGF-β co-treatment. HeLa cells co-transduced with adenoviruses carrying the Twist1 and shNF-κBp65 genes were co-administered TNF-α and TGF-β, with or without ChR(10.0μM). Then, cell morphology (A, scale bar, 50 μm), E-cadherin and N-cadherin protein amounts (B), NF-κBp65 and Twist1 protein levels (C), cell migration (D) and self-renewal (E, scale bar, 200 μm) abilities, and the protein expression levels of Bmi1, Sox2 and Oct4(F), as well as CD133, ALDH1, and CD44 (G) were determined. β-actinserved as an internal reference for immunoblots. cDNA, cells transduced with adenoviruses expressing Twist1; cDNA+shRNA, cells co-transduced with adenoviruses harboring the Twist1 and shNF-κBp65 genes.*$p<0.05$ vs shNF-κBp65. #$p<0.05$ vs shNF-κBp65 with ChR(10.0μM) treatment.

in Fig. 1 and Supplementary Fig. 1. Similar to the results reported by Li CW. *et al.* TNF-α induced nuclear translocation of NF-κBp65 at 0.5h, whereas the nuclear expression of Twist1 began to increase at 1 hr after treatment following prolonged administration of TGF-β, suggesting TNF-α triggers a dynamic interaction between nuclear translocation of NF-κBp65 and nuclear expression of Twist1.These findings provide novel insights into the signaling pathways of cervical cancer stem like cells, introducing a novel strategy for the treatment of human cervical cancer.

ChR, an active natural bioflavonoid, has a biological importance due to its multiple effects such as antioxidant, anti-inflammatory, antimicrobial, anti-allergic, and anti-tumor effects [29-32]. In addition ChR downregulates NF-κB and its antiapoptotic target gene c-FLIP-L [40]. Therefore, we used different concentrations of ChR (5.0, 10.0 and 20.0μM) to treat HeLa cells administered TNF-α combined with TGF-β. The results showed that ChR dose-dependently downregulated NF-κBp65 and Twist1, and suppressed EMT and CSLC features

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in HeLa cells co-treated with TNF-α and TGF-β, as illustrated by Fig. 2. We unexpectedly found that ChR inhibited nuclear translocation of NF-κBp65 at 3h, whereas the nuclear expression of Twist1 began to reduce at 6h after treatment. Notably, ChR significantly reduced DNA binding activity of NF-κBp65 at 3 h, 6 h, 12 h and 24 h, as compared with the same time points in vector (0.1% DMSO) control. Therefore, the further investigation of whereby ChR reduces nuclear expression and DNA binding activity of NF-κBp65 is conceivable. These findings suggest that the multiple biological activities of ChR may be involved in regulating the NF-κBp65/Twist axis.

It has been shown that TNF-α associated EMT requires NF-κB-modulated Twist1 upregulation at the gene level [41], which indicates Twist1 is a key downstream effector of NF-κB. In this study, NF-κBp65 silencing or overexpression accordingly altered Twist1 protein levels, and increased or attenuated the effects of ChR; however, Twist1 silencing or overexpression did not affect NF-κBp65 expression but promoted or reduced the effects of ChR. These findings provided convincing evidence that NF-κBp65 is located upstream of Twist1. To demonstrate the involvement of NF-κB/Twist signaling, we performed a functional rescue assay using Twist1 gene and shNF-κB co-transduction. As shown above, Twist1 overexpression almost totally reversed the effects of co-treatment with shNF-κB and ChR, as shown in Fig. 7.

Conclusion

The present study revealed that ChR inhibits proinflammatory factor associated EMT phenotype and CSLC features in HeLa cells by blocking NF-kB/Twist signaling. Although nude mouse xenograft and clinical studies were not performed, these *in vitro* findings may provide a basis for developing promising adjunctive therapeutics for human cervical cancer.

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Disclosure Statement

All authors declare that they have no any conflicts of interest.

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