

Niaoduqing Granules Inhibits TGF- β 1-induced Epithelial-Mesenchymal Transition in Human Renal Tubular Epithelial HK-2 Cells

Chu-Ying Huo

Southern Medical University

Hua-Yi Yang

Southern Medical University

Wei-Min Ning

Southern Medical University

Lin-Zhong Yu

Southern Medical University

Chun-Lin Fan

Jinan University College of Pharmacy

Jing-Yu Quan

Southern Medical University

Li-Er Deng

Dongguan Hospital of Traditional Chinese Medicine

Zhi-Ling Yu

Hong Kong Baptist University

Jun-Shan Liu

Southern Medical University

Hui-Hui Cao (✉ huizizheng@hotmail.com)

Southern Medical University

Research

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Abstract

Background: Chronic renal failure (CRF) is a worldwide public health burden. Niaoduqing granules (NDQ) is widely used for CRF treatment in China. However, the underlying mechanism of NDQ is not fully studied. This study is aimed to investigate whether NDQ ameliorate CRF by inhibiting TGF- β 1-induced EMT in human renal tubular epithelial HK-2 cells.

Methods: MTT assay and colony formation assay were used to investigate the cytotoxicity of NDQ in HK-2 cells. Morphological changes of HK-2 cells after TGF- β 1 or/and NDQ treatment were observed under a microscope. Wound-healing, migration and invasion assays were performed to determine the cell movement, migratory and invasive abilities, respectively. Western blot analysis was carried out to examine the protein levels of TGF- β type I receptor (T β RI) and EMT-associated factors. Fluorescence confocal microscopy was applied to observe the organization of F-actin.

Results: NDQ suppressed T β RI expression dose-dependently. NDQ inhibited TGF- β 1-stimulated EMT in HK-2 cells, supported by the evidences that NDQ prevented morphology change, attenuated cell migration and invasion, downregulated EMT factors and reorganized F-actin distribution in TGF- β 1-stimulated HK-2 cells.

Conclusions: NDQ attenuates chronic renal failure which may be associated with inhibition of T β RI expression and EMT process.

Background

Chronic renal failure (CRF), a common result of chronic kidney disease (CKD), has become a health problem in the world [1]. Renal fibrosis is the main pathological feature of CRF, and inhibition of renal fibrosis is regarded as an effective therapeutic approach for CRF [2].

Emerging evidence implicates that epithelial-mesenchymal transition (EMT) of renal tubular promotes renal fibrosis. EMT is a process that epithelial cells lost their epithelial characteristics and transform into mesenchymal cells with mesenchymal characteristics. Transforming growth factor- β 1 (TGF- β 1) is known as a trigger of EMT and plays a pivotal role in renal fibrosis [3, 4]. Activation of TGF- β 1 mediates EMT and eventually leads to fibrosis in human renal, while inhibition of TGF- β 1 activation alters the EMT process and reduces fibrosis [5], indicating that blockade of TGF- β 1 signaling is a therapeutic strategy for CRF.

Niaoduqing granules (NDQ), a Chinese medicine preparation, which included *Radix et Rhizoma Rhei*, *Radix Astragali*, *Cortex Mori*, *Radix Sophorae Flavescens*, *Rhizoma Atractylodis macrocephalae*, *Poria*, *Radix Paeoniae Alba*, *Radix Polygoni Multiflori*, *Radix Salviae Miltiorrhizae* and *Herba Plantaginis* (see Additional file 1), is frequently used for CRF treatment. Clinical studies showed that NDQ reduced serum creatinine and blood urea nitrogen, improved renal function [6], and also alleviates CKD progression in patients with moderate-to-severe renal insufficiency efficiently [7]. In animal models, NDQ was found to

reduce tubulointerstitial fibrosis and relieve CKD in rat, which were related with the modulation of TGF- β and erythropoietin signaling pathways [6, 8, 9]. However, it's still unclear that if NDQ attenuates CRF by inhibiting TGF- β 1-induced EMT.

In this study, we first demonstrated that NDQ downregulated the expression level of TGF- β receptor I (T β RI) in human renal tubular epithelial HK-2 cells, inhibited TGF- β 1-induced EMT and dysregulated the distribution of filamentous actin (F-actin), which may account for the treatment of CRF by NDQ.

Methods

Drug and reagents

NDQ [National drug standard No: WS3-229(Z-033)-2000(Z)] was obtained from Guangzhou Kangchen Pharmaceutical Co., Ltd. (Guangzhou, China), and granules were dissolved in PBS at a concentration of 1.25 g/ml.

TGF- β 1 peptides was purchased from Biosynthesis biotechnology (Beijing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) was obtained from Thermo Fisher Scientific (Waltham, USA). 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was obtained from Beyotime Biotechnology (Shanghai, China). Antibodies against Vimentin, N-cadherin, snail and slug were purchased from Cell Signaling Technology (Danvers, USA). Antibodies against TGF- β receptor I was purchased from abcam (Cambridge, UK). Rhodamine phalloidin was purchased from cytoskeleton (Denver CO, USA). Other reagents were obtained from Sigma-Aldrich (St. Louis, USA).

Cell line and cell culture

Human kidney tubular epithelial cell line HK-2 was obtained from Otwo Biotech (Shenzhen) Inc. (Shenzhen, China).

HK-2 cells were cultured in RPMI-1640 with 10% fetal bovine serum (FBS, Sijiqing, Hangzhou, China) and 1% penicillin-streptomycin (Gibco, USA) in a humidified incubator with 5% CO₂ at 37 °C.

Cell viability assay

The viability of NDQ in HK-2 cells was determined by MTT assay as previously described [10]. Cells (10⁴/well) were seeded in 96-well plates for 24 h and then exposed to different concentrations of NDQ for 24 h, 48 h and 72 h, respectively. After treatment, the supernatant was discarded and 30 μ l of MTT solution (5 mg/ml) was added and incubated for another 4 h at 37 °C. And then, the supernatant was discarded and the purple formazan crystals were dissolved in 100 μ l of DMSO and the absorbance was measured at 570 nm by a microplate reader (Multiskan FC, Thermo Scientific, USA).

Colony formation assay

HK-2 cells (300/well) were seeded in 6-well plates for 24 h and then treated with NDQ at different concentrations. After incubated for 24 h, cells were washed with phosphate-buffered saline (PBS), and cultured in fresh medium for 10 days. Then, cells were fixed in 75% alcohol at 4 °C and stained with Giemsa dye [11].

Cell morphological observation

HK-2 cells (10^5 /well) were seeded in 6-well plates and incubated for 24 h. Cells were then serum-starved for 24 h followed by NDQ treatment for 24 h with or without TGF- β 1. After that, cells were washed with PBS and morphological changes were observed under a microscope (IX 53, Olympus, Tokyo, Japan).

Cell migration and invasion experiment

For wound healing experiment, HK-2 cells (3×10^5 /well) were seeded in 6-well plates for 24 h and serum-starved for another 24 h. Cells were then scratched in a straight line with 200- μ l pipette tips and treated with different concentrations of NDQ in the presence or absence of TGF- β 1. Images of HK-2 cells were acquired with a microscope (IX 53, Olympus, Tokyo, Japan) at 0 h, 12 h and 24 h, respectively.

In the Transwell migration assay, HK-2 cells (10^5 /well) were seeded in Transwell chamber with RPMI-1640 medium for 24 h and 400 μ l of RPMI-1640 medium containing 10% FBS was added into the bottom chamber. Cells were then incubated with NDQ for 24 h under TGF- β 1-stimulated or unstimulated conditions, and chambers were fixed using 75% alcohol before stained with Giemsa dye. After that, cells remaining on the upper surface of the membrane were removed by wiping and the images of migrated cells were obtained under a microscope (IX 53, Olympus, Tokyo, Japan). The invasion assay shared the same method with the Transwell migration assay, the only difference was, matrigel was applied in the Transwell chamber before cells were seeded.

Western blot analysis

HK-2 cells (3×10^5 dish) were seeded in 60 mm dishes for 24 h and serum-starved for another 24 h. After treated with different concentrations of NDQ for 48 h with or without TGF- β 1 stimulation, cells were harvested and washed with cold PBS, and then lysed for 15 min at 4 °C with RIPA buffer (0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM sodium orthovanadate, 0.1 mM dithiothreitol and phosphatase inhibitor). After centrifugation at 13,500 rpm for 15 min, supernatants were collected as total protein. The protein concentrations were determined by a BCA protein assay kit. The method of Western blotting was performed as previously described [12]. Protein levels were quantified by ImageJ 1.4.3 (National Institutes of Health, USA).

F-actin fluorescence confocal microscopy

HK-2 cells (3×10^5 dish) were seeded in confocal dishes for 24 h. After incubated in serum-free medium for 24 h, cells were exposed to NDQ for another 24 h in the presence or absence of TGF- β 1. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonX-100 in PBS. After blocked with 5% bull serum albumin (BSA) for 15 min at room temperature (RT), cells were incubated with 100 μ l of rhodamine phalloidin (70 nM) for 1 h at RT. Subsequently, cells were washed and counterstained with 100 μ l of DAPI. The fluorescence was observed by confocal microscopy (LSM800, Carl Zeiss, Oberkochen, Germany).

Statistical analysis

All data were expressed as the means \pm SEM and analyzed by Graphpad Prism 5.0 software (Graphpad software Inc., USA). Tukey's test was used for multiple comparison. The values were considered statistically significant when $P < 0.05$.

Results

Cytotoxicity of NDQ in HK-2 cells

To determine the cytotoxicity of NDQ in HK-2 cells, MTT assay and colony formation assay were used. The results showed that NDQ inhibited the proliferation of HK-2 cells in a dose- and time-dependent manner (Fig. 1A). However, NDQ exerted no significant cytotoxicity in HK-2 cells at the dosage below 5 mg/ml (Fig. 1B), and data from colony formation assay further confirmed this result (Fig. 1C). We therefore use NDQ with the dosage of 5 mg/ml for further study.

NDQ downregulates the expression level of T β RI in HK-2 cells

TGF- β 1 signaling is considered to have a pivotal role in renal fibrosis and EMT process [5]. It is reported that the activation of TGF- β 1 signaling is initiated by the binding of TGF- β 1 ligand to T β RII and T β RI [13]. Therefore, we wonder whether NDQ affect the expression of T β RI. HK-2 cells were exposed to indicated concentrations of NDQ for 48 h, followed by Western blot analysis. As shown in Fig. 2, NDQ downregulated the expression level of T β RI in a dose-dependent manner. Our finding suggested that NDQ may blocked TGF- β 1 signaling through repression of T β RI.

NDQ attenuates the morphological changes in TGF- β 1-stimulated HK-2 cells

T β RI is critical in TGF- β 1 signaling, which mediates downstream and leads to EMT [14]. Based on the evidence that NDQ downregulated the expression level of T β RI, we suspected NDQ exerts inhibitory effects on TGF- β 1 signals and subsequently suppresses EMT in renal epithelial cells. TGF- β 1, a well-known inducer of EMT, was added and incubated with HK-2 cells for 24 h after serum-starved for 24 h to establish a model of EMT in HK-2 cells. The morphology of HK-2 cells was changed and became slender and fibroblast-like after simulated with TGF- β 1 (Figure 3, red arrow), while NDQ prevented cells from these morphological changes.

NDQ inhibits TGF- β 1-induced EMT in HK-2 cells

To verify the effect of NDQ on TGF- β 1-induced EMT, we determined the expression levels of EMT-associated protein, including N-cadherin, vimentin, slug and snail. Our data showed that TGF- β 1 upregulated the expression levels of N-cadherin, vimentin, slug and snail, while NDQ prevented the induction of EMT by TGF- β 1, with downregulation of the N-cadherin, vimentin, slug and snail expression levels (Figure 4).

NDQ inhibits cell movement, migration and invasion in TGF- β 1-stimulated HK-2 cells

When cells undergo EMT, cell adherence is attenuated and cell mobility is enhanced [15]. To investigate the effects of NDQ on cell movement in HK-2 cells, wound-healing assay was performed. We found that TGF- β 1 promoted cell movement, nevertheless, NDQ inhibited the TGF- β 1-induced cell movement in HK-2 cells (Figure 5A). Further evidences were provided by Transwell migration assay and invasion assay. As shown in Figure 5B, TGF- β 1 remarkably enhanced the migratory ability of HK-2 cells. However, NDQ treatment reduced cell migration. As such, the invasion assay result also demonstrated that NDQ reversed cell invasion induced by TGF- β 1 in HK-2 cells (Figure 5C).

NDQ reorganized the arrangement of F-actin in TGF- β 1-stimulated HK-2 cells

F-actin, an important component of cytoskeleton, involves in cell migration and invasion [16]. We subsequently detected the arrangement of F-actin followed by NDQ treatment. Rhodamine phalloidin was applied to visualize the distribution of the F-actin in HK-2 cells. As shown in Figure 6, TGF- β 1 increased the expression and longitudinally distribution of F-actin. Compared to TGF- β 1-stimulated cells, NDQ treatment decreased the intensity and reorganized the distribution of F-actin in HK-2 cells. These data further validated that NDQ attenuated TGF- β 1-induced EMT in HK-2 cells.

Discussion

As a burden on global health, CRF is mainly featured by tubulo-interstitial fibrosis, regardless of the initial etiology of the renal disease. Hence, renal fibrosis is a universal feature of CRF and contributes dramatically to end-stage renal failure [17]. There are two main therapeutic strategies for CRF to improve renal function: dialysis and renal transplantation. Nevertheless, these therapies lead to the increased risk of cardiovascular problem [18]. Clinical observation showed that NDQ dramatically alleviate renal dysfunction and tubulointerstitial fibrosis for CKD patients at stage III to stage IV [19]. Previous studies about CRF treatment tended to choose NDQ as a positive medicine [20–23], indicating its certain efficacies. In this study, we demonstrate that NDQ contributes to CRF by downregulation of T β RI expression level, inhibiting the EMT processing, and rearranging the F-actin organization.

Renal fibrosis is a characteristic by which CKD develops into CRF. Previous studies have reported that EMT is a phenotypic conversion that has emerged as a critical role in renal fibrosis and contributes to the progression of CRF [24]. TGF- β 1 has a predominant role in the progress of CRF. Accumulating evidences have pointed out that TGF- β 1 activates its downstream to contribute to EMT and renal fibrosis. TGF- β receptors, including T β RI and T β RII, participates in TGF- β signals. At the cell membrane, TGF- β 1, serving as a ligand, first binds to T β RII, and subsequently recruits T β RI and induces its activation. After receptor binding, TGF- β 1 exerts its cellular functions and promotes the EMT process [25]. Hence, the activation of T β RI is pivotal in TGF- β 1 signaling. It was reported that T β RI downregulation led to EMT suppression in TGF- β -stimulated nasal epithelial cells [14]. Previous research also suggested that suppressed TGF- β 1/TGF- β type I receptor/Smads signaling activation *in vivo and in vitro*, suppressing the EMT and collagen deposition to alleviate renal fibrosis that the inhibition of renal fibrosis was regarded as an effective therapeutic approach for CRF [26]. In this study, we first demonstrated that NDQ inhibits the expression level of T β RI in human renal tubular epithelial HK-2 cells, while previous study revealed that NDQ downregulated the expression level of T β RI in CRF rats [9]. We will further investigate if NDQ inhibit the membrane T β RI levels, and the effects of NDQ on T β RI transcription, translation, or post-translation.

The occurrence of EMT leads to the loss of epithelial markers of renal tubular cells, the development of invasive and metastatic in cells and the increased of mesenchymal features in morphology, such as spindle-cell-like morphology. Moreover, during the EMT process, the expression of the epithelial markers, including E-cadherin, is downregulated, whereas the mesenchymal markers, such as N-cadherin, Snail, Slug, and Vimentin are increased. N-Cadherin is the main contributors to reduced cell–cell adhesion in epithelial tissues and eventually lead to cell de-differentiation, tissue disorder and increased cell invasion capacity, ultimately, to metastasis. Vimentin is an intermediate filament protein and acts as a cytoskeletal element, which is also known as a mesenchymal marker. Besides, EMT-inducing transcription factors include Snail and Slug. Especially, Snail is associated with the initial cell-migratory phenotype and is regarded as an early marker of EMT [27, 28]. In our research, TGF- β 1 alternated the morphology of HK-2 cells, while NDQ prevented cells from these changes. It was also found that NDQ attenuated cell migratory and invasive abilities as well as inhibited TGF- β 1-induced upregulation of EMT markers including N-cadherin, Snail, Slug, and Vimentin in HK-2 cells, indicating that NDQ inhibited TGF- β 1-induced EMT in HK-2 cells. These results were in agreement with a previous study by Lu *et al* [29]. However, Lu *et al*/ used rat serum containing NDQ for the mechanistic study and our research is the first

time use NDQ directly in human renal tubular epithelial cells and demonstrates its inhibitory effects on EMT.

For years, EMT is considered as a binary process with cells individually detach from tissue. However, recent studies demonstrate epithelial cells and mesenchymal cells are co-exist as a cluster in many cases, which is called hybrid E/M phenotype, and this phenotype presents more migratory and invasive properties [30]. This concept indicates an alternative EMT process in NDQ intervention. In this regard, more experiments will be needed to explore the extent of NDQ-induced EMT suppression. Recent research also suggested that transcription factors like GRHL2 or OVOL2 may be the crucial EMT drivers [31, 32], whether NDQ inhibit the expression levels of the other regulators is unknown and deserves further exploration.

F-actin is an important protein of muscle thin filaments, which constitute the eukaryotic cytoskeleton. Remodeling of F-actin facilitates cell migration and invasion [16, 33]. At the final process of EMT, the cells reorganize their cortical actin cytoskeleton to enable dynamic cell elongation and directional motility [34]. Thus, F-actin reorganization directly controls cell migration/invasion. Since EMT is characterized by increased cell migration/invasion and actin stress fiber formation, F-actin plays an important role in renal fibrosis. Our results showed that NDQ significantly reduced F-actin intensity and remodeled its organization in HK-2 cells. These evidences strongly suggest that NDQ suppresses CRF through cell migration and invasion via reorganizing the arrangement of F-actin.

It should be pointed out that once T β RI is activated, the canonical Smad signaling and non-canonical Smad pathways are generally activated to regulate gene transcription for performing physiological functions. Although the inhibition of the protein expression of T β RI by NDQ was demonstrated, the specific pathways that it regulates need further exploration [35–37].

Conclusion

In conclusion, we first demonstrated that the renoprotective effect of NDQ is partially attributed to the downregulation of T β RI, which account for the inhibition of TGF- β 1-induced EMT in HK-2 cells (Fig. 7). This study provides a pharmacological basis for the clinic use of NDQ in the treatment of CRF.

Abbreviations

CRF, chronic renal failure; CKD, chronic kidney disease; EMT, epithelial-mesenchymal transition; TGF- β 1, transforming growth factor; NDQ, niaoduqing granules; T β RI, TGF- β receptor I; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; ECL, enhanced chamiluminescence; RT, room temperature; F-actin; filamentous actin; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; BSA, bull serum albumin.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publish

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding upon request.

Competing interests

No conflicts of interest were declared by the authors.

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Authors' contributions

CYH and HYY carried out the experiments, data analysis and manuscript writing. WMN performed the statistical analysis and prepared figures. LZY and CLF participated in experimental work and results writing. JYQ and LED carried out the experiments and discussion writing. ZLY, JSL and HHC designed and supervised the study. All authors have read and agreed to publish this manuscript.

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Figures

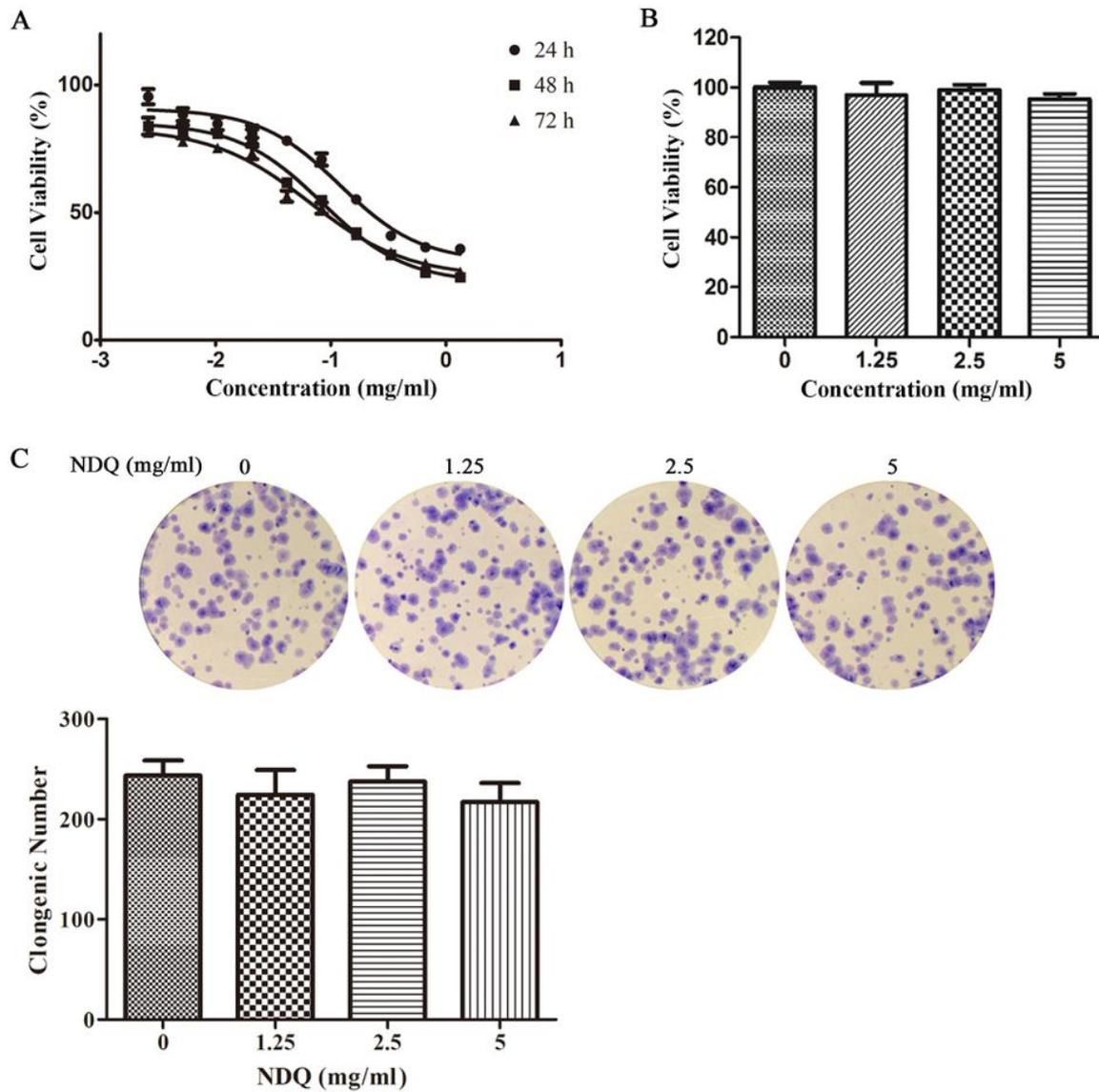


Figure 1

Cytotoxicity of NDQ on HK-2 cells. (A) NDQ inhibited the proliferation of HK-2 cells in a time- and dose-dependent manner. (B) NDQ showed no significant effect on the viability of HK-2 cells at the dosage below 5 mg/ml. (C) NDQ had no obvious long-term cytotoxicity on HK-2 cells at the dosage below 5 mg/ml.

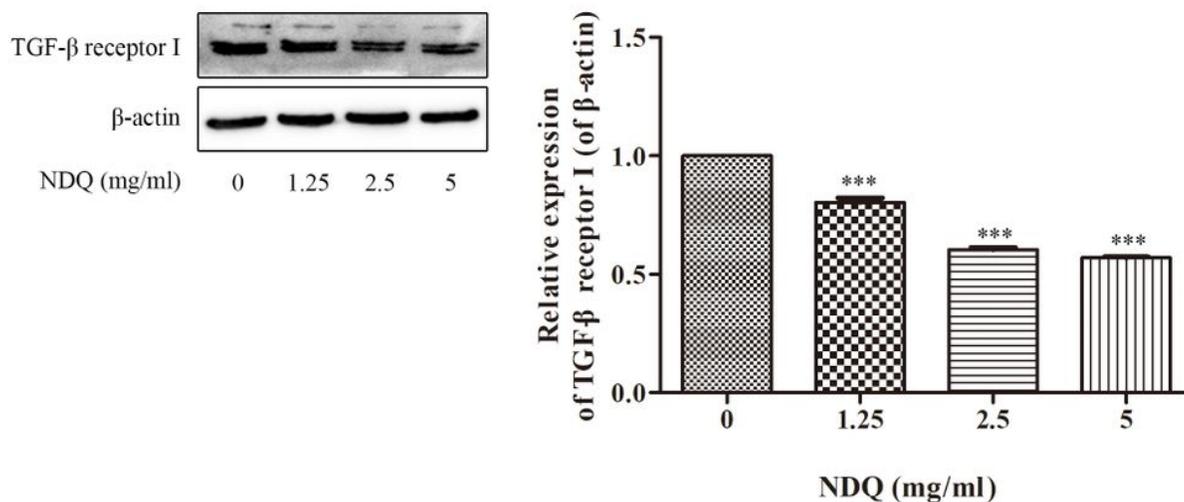


Figure 2

NDQ downregulates TβRI expression in HK-2 cells. HK-2 cells were treated with indicated concentration of NDQ and the expression level of TβRI was determined by Western blot analysis. The representative figures (left) and the relative expression levels analyzed by ImageJ 1.4.3 software (right) were shown. ***P < 0.001, versus Control group. One-way ANOVA, post hoc comparisons, Turkey's test. Columns, means; error bars, SEM.

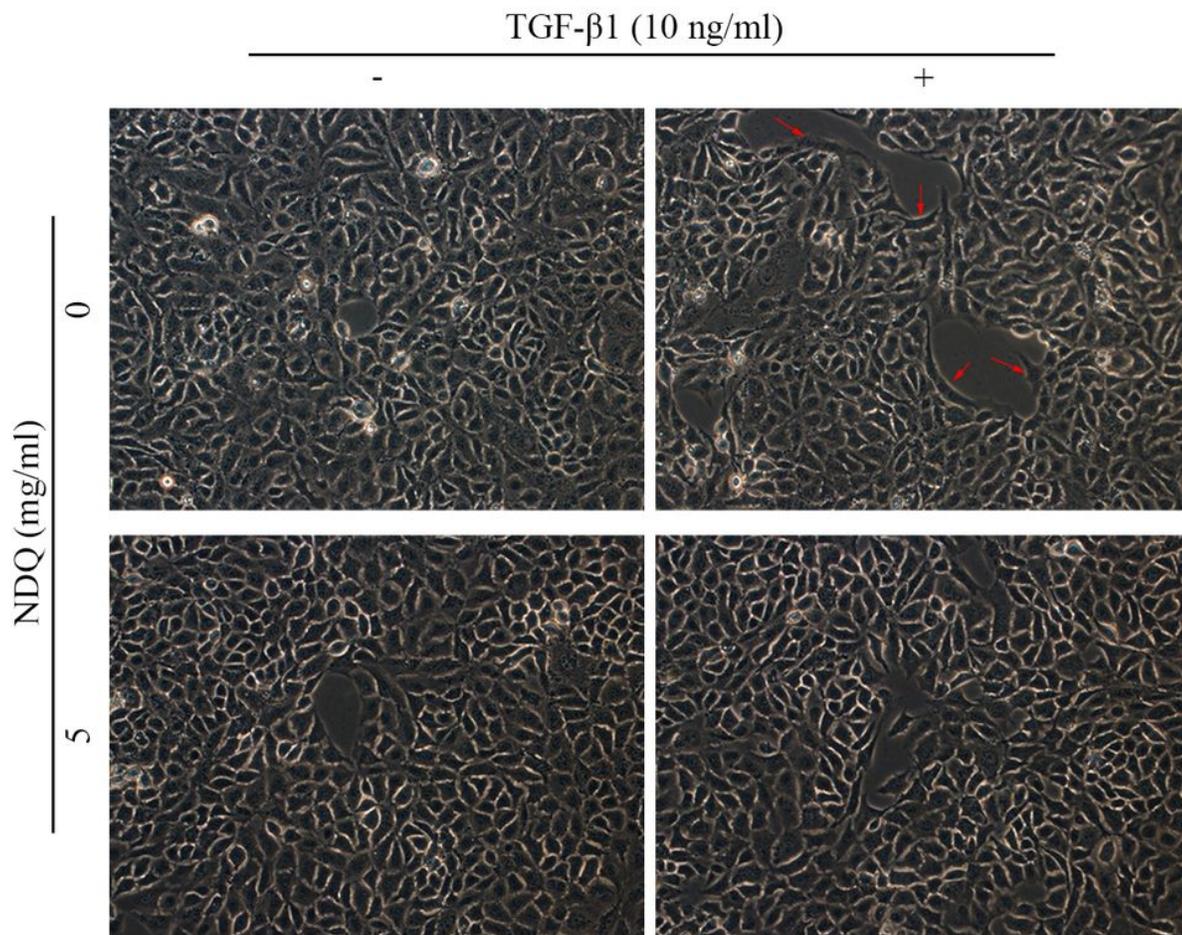


Figure 3

Morphology changes in HK-2 cells. Cells were serum-starved for 24 h and then exposed to vehicle or NDQ for 24 h with or without TGF-β1 stimulation. Fibroblast-like morphology changes were observed in TGF-β1-stimulated HK-2 cells (red arrow), and these changes were attenuated after NDQ treatment.

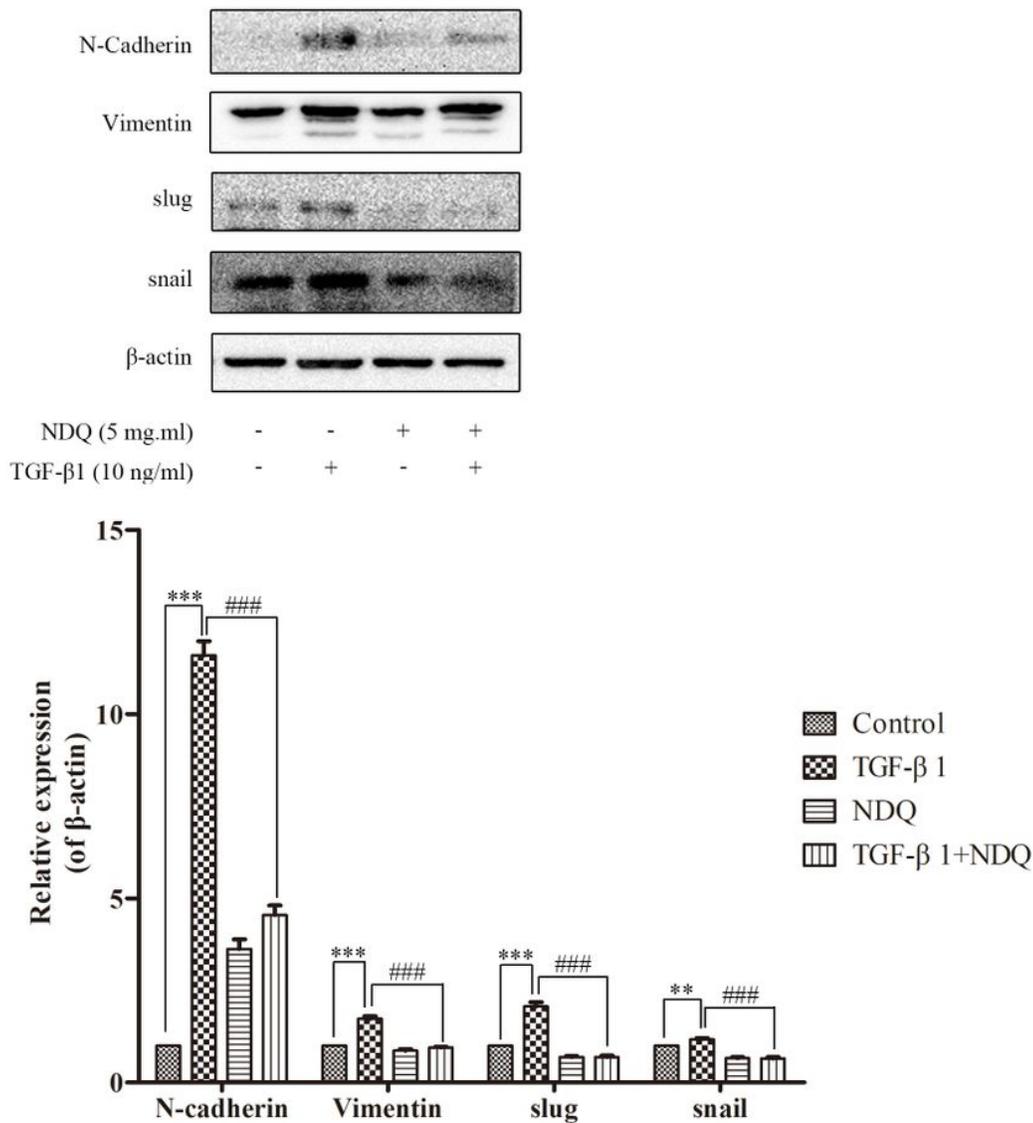


Figure 4

NDQ inhibits TGF- β 1-induced upregulation of EMT-associated factors in HK-2 cells. HK-2 cells were serum-starved for 24 h and then treated with indicated concentration of NDQ for 48 h in the presence or absence of TGF- β 1. Expression levels of N-cadherin, vimentin, snail and slug were examined by Western blotting using specific antibodies. The representative figures (upper) and the relative expression levels analyzed by ImageJ 1.4.3 software (bottom) were shown. * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$, versus Control group. ### $P \leq 0.01$ and #### $P \leq 0.001$, versus TGF- β 1 group. One-way ANOVA, post hoc comparisons, Turkey's test. Columns, means; error bars, SEM.

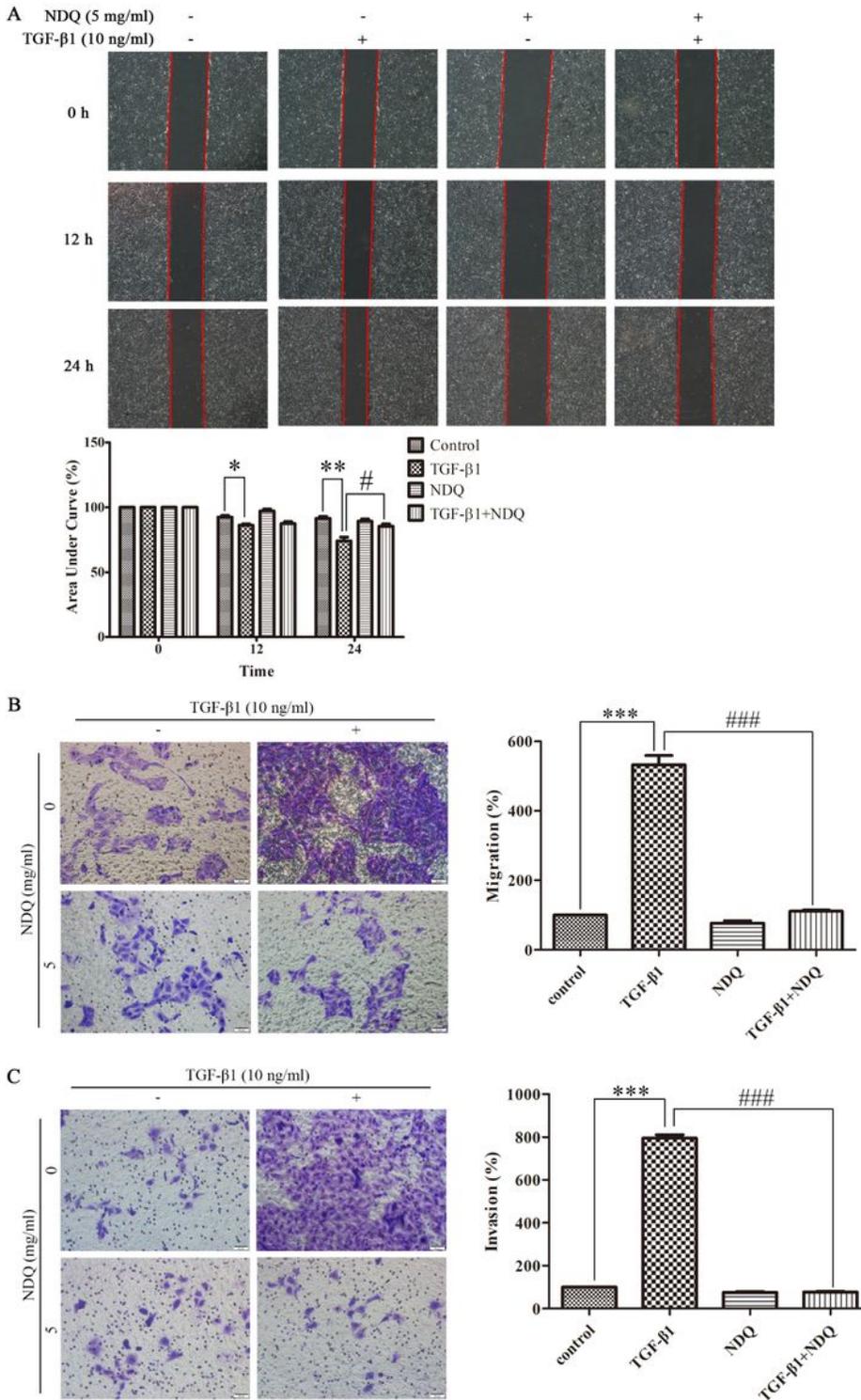


Figure 5

NDQ attenuates cell migration and invasion in TGF- β 1-stimulated HK-2 cells. Cell migration was measured by wound-healing (A) and Transwell migration assay (B), and invasive ability of HK-2 cells was measured by Transwell invasion assay (C). Relative migrated areas were quantified by ImageJ 1.4.3 software. Quantification of migrated and invasive cells were analyzed by Graphpad Prism 5.0 software.

***P \leq 0.001, versus Control group. ###P \leq 0.001, versus TGF- β 1 group. One-way ANOVA, post hoc comparisons, Turkey's test. Columns, means; error bars, SEM.

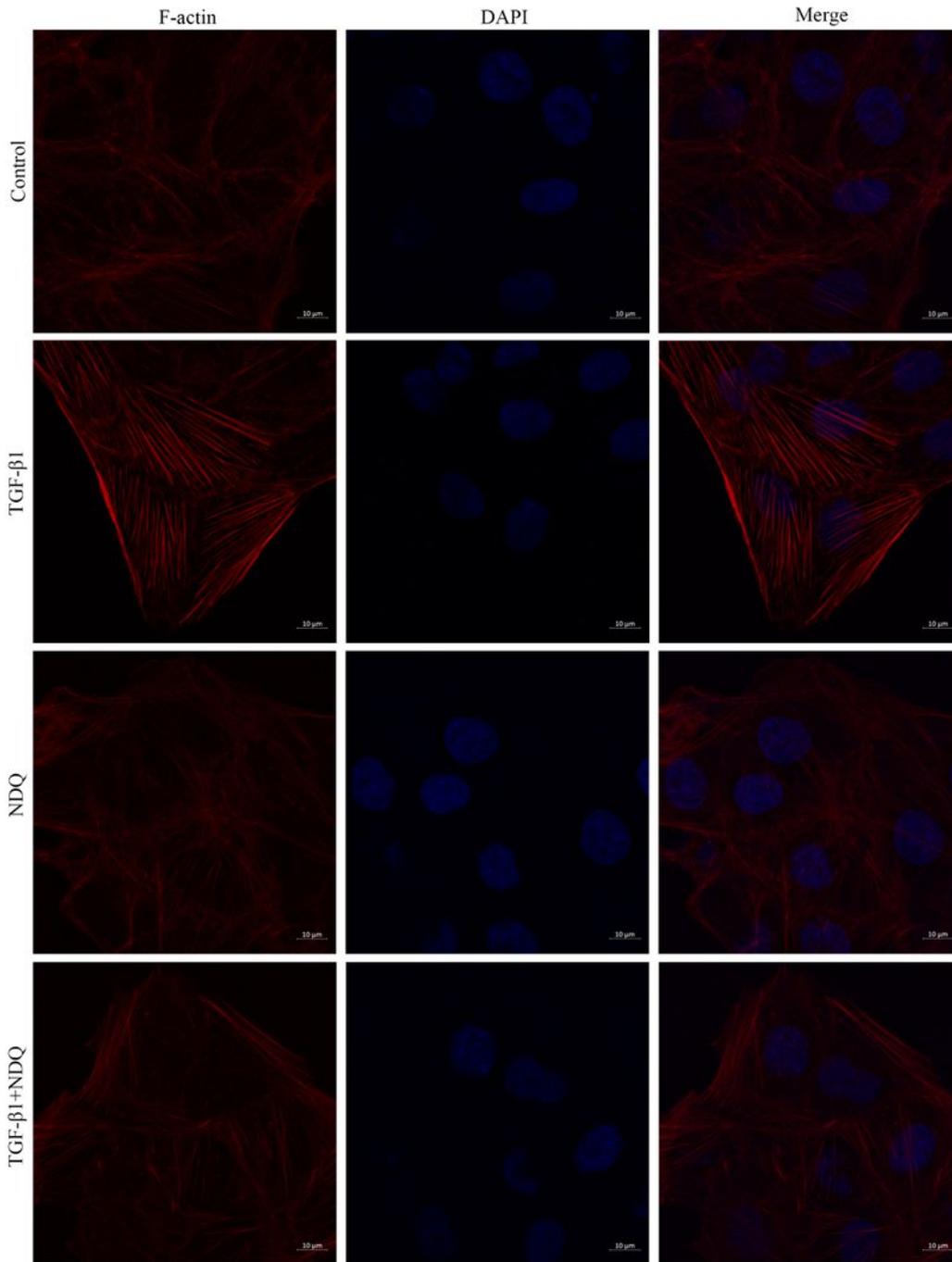


Figure 6

NDQ reorganized the distribution of F-actin. HK-2 cells were serum-starved for 24 h and then exposed to vehicle or NDQ (5 mg/ml) in the presence or absence of TGF- β 1 for another 24 h, the distribution of F-

actin was visualized by confocal after rhodamine phalloidin staining. Left: F-actin, middle: DAPI, right: merge.

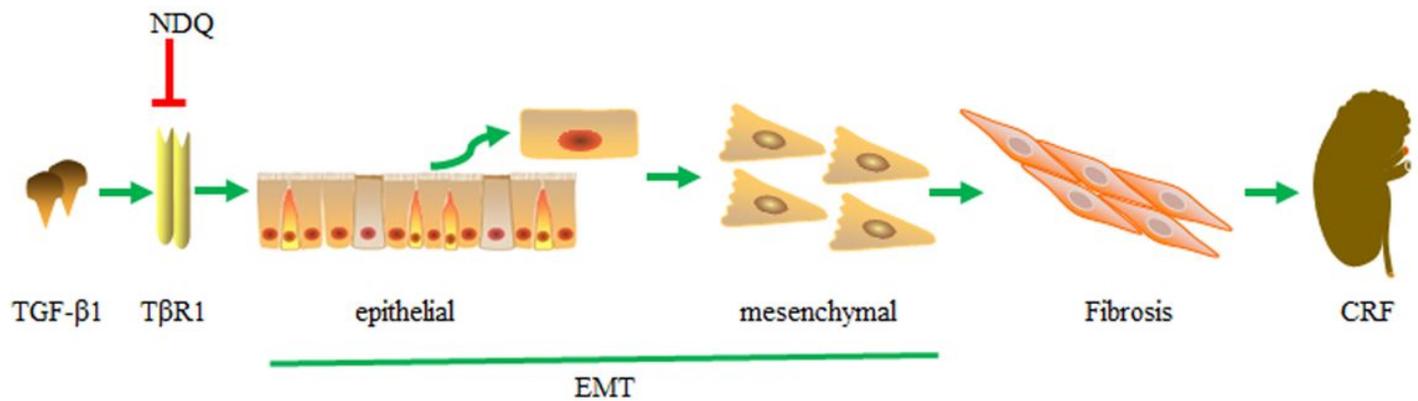


Figure 7

The underlying mechanism of NDQ on preventing epithelial cells from TGF-β1-induced EMT. → refers to a promoting effect and ⊥ refers to a blockage effect.

Supplementary Files

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- [supplement1.docx](#)