Original Article
Knockdown of high-mobility group nucleosome-binding protein 2 increases uropathogenic *Escherichia coli* invasion in human bladder epithelial cells through promoting actin cytoskeleton polymerization

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Abstract: High-mobility group nucleosome-binding protein 2 (HMGN2) is a non-histone nuclear protein that can bind nucleosomes and regulate chromosome architecture and gene transcription. Our previous results indicate that intranuclear HMGN2 acts as a positive modulator of NF-κB signalling to promote LPS-induced β-defensin expression. Meanwhile, HMGN2 can be released extracellularly and suppress of bacterial invasion into bladder epithelial cells (BECs). In this study, we report that intranuclear HMGN2 was also involved in the invasion of uropathogenic *E. coli* (UPEC) into BECs. HMGN2 knockdown resulted in elevated UPEC invasion into 5637 and T24 BECs. And HMGN2 gene silencing could induce actin polymerization, which is essential for α3/β1 integrins mediated UPEC internalization into BECs. However, the expressions of α3/β1 integrins and the phosphorylation of FAK and Src were not obviously affected by HMGN2 knockdown. Interestingly, increased expression of Rac1-GTPase was observed in HMGN2 silent BECs, which lead to actin polymerization and UPEC invasion. Furthermore, extracellular regulated protein kinases 1/2 (ERK1/2) was mostly and p38 mitogen-activated protein kinase (MAPK) was partially involved in HMGN2 silenting-mediated promotion of UPEC invasion, as confirmed with chemical inhibitors of ERK1/2 and p38 MAPK. These data suggested a potential role of HMGN2 in innate immune response during UPEC induced bladder infections.

Keywords: HMGN2, UPEC invasion, actin polymerization, Rac1-GTPase, MAPKs

Introduction

Urinary tract infections (UTIs), a very common and frequently recurrent bacterial disease, affect more than 150 million people annually worldwide, resulting in approximately 14 million hospital visits each year, and an estimated total cost of 6 billion dollars in direct health care [1]. The invasive pathogen uropathogenic *Escherichia coli* (UPEC) are the major causative agents of UTIs in healthy adults, mainly causing acute bladder infection (cystitis) and kidney infection (pyelonephritis), and resulting in severe chronic renal failure or bacteremia/sepsis [2]. To establish a successful UTI, UPEC must adhere to the superficial umbrella cells of bladder epithelium, followed by invading into the cytosolic milieu of these cells. Bacterial invasion follows the binding of type 1 fimbriae adhesin FimH to the mannosylated glycoproteins (including uroplakin 1a and α3/β1 integrins) found on the apical surface of superficial bladder epithelial cells (BECs) [3, 4]. FimH-mediated bacterial invasion of host BECs requires rearrangement of actin cytoskeleton. A number of signaling molecules and adaptor proteins known to regulate actin dynamics, including Rho GTPases, focal adhesion kinase (FAK), Src family kinases and mitogen-activated protein kinases (MAPKs), have been implicated in the bacterial invasion process [5, 6]. Hence, modulating the expressions or activities of these mentioned factors may interfere with the UPEC invasion in bladder epithelium.

High mobility group nucleosome-binding proteins (HMGNs) are small nonhistone nuclear proteins. HMGNs have the unique ability to bind to nucleosomes, which in turn control the chromosome architecture and dynamics, modify gene transcription, and regulate DNA repair, cell differentiation, and ontogenic development [7]. HMGN2, a major member of HMGNs, is ubiquitously present in almost all embryonic and adult tissues of higher vertebrates [8]. Similar to other HMGN proteins, HMGN2 also exerts diverse nuclear factor-like functions inside the nucleus [7]. Although long viewed as general transcriptional activators on chromatin templates, it is now appreciated that HMGNs are specific transcription modulator of gene expression, such as glycine transporter (*GLY1*) [9], heat shock protein 70 (*HSP70*) [10], estrogen-regulated genes *TFF1* and *FOS* [11], and signal transducer and activator of transcription 5α (*STAT5α*) [12]. Recently, our results indicate that intranuclear HMGN2 acts as a positive modulator of NF-κB signalling to promote LPS-induced expression of β-defensin, a key mediator of innate immune responses [13]. Meanwhile, HMGN2 can be released from human peripheral blood mononuclear cells initiated by interleukin-2 (IL-2) and phytohemagglutinin (PHA) stimulation [14]. And the exogenous HMGN2 has been found to be antimicrobial polypeptide against fungi, bacteria and viruses [15]. Furthermore, exogenous HMGN2 also inhibits the bacterial invasion in T24 BECs through regulating reorganization of actin cytoskeleton [16]. Therefore, HMGN2 may be an important regulator in mucosal innate immune responses during infection. However, the role and mechanism of intranuclear HMGN2 in bladder infection is yet unknown. We have also found that knockdown of HMGN2 in A549 airway epithelial cells led to significant expression changes in some cytoskeletal rearrangement and cell adhesion related genes, including integrins, Rho GTPase and MAPKs [13]. Thus, in the present study, we hypothesize that intranuclear HMGN2 might act as a specific modulator that interferes the bacterial adhesion and invasion in BECs by regulating the cytoskeletal rearrangement and cell adhesion related factors.

Materials and methods

Cell lines and cell culture

Human bladder epithelial cell (BEC) lines T24 (ATCC HTB-4) and 5637 (ATCC HTB-9) were obtained from the Cell Bank of the Chinese Academic of Sciences (Shanghai, China), and cultured in RPMI 1640 medium (HyClone, Beijing, China) with 10% fetal bovine serum (FBS, FuMeng Gene Co., Ltd., Shanghai, China) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Beyotime, Haimen, China) in an atmosphere with 5% CO_2 at 37°C.

Bacterial strains and culture conditions

The uropathogenic *E. coli* (UPEC) strain J96 (ATCC 700336) was used for cell infection. Bacteria were grown to logarithmic phase in a static Luria-Bertani (LB) medium at 37°C. Bacteria were collected by centrifugation at 3600 rpm/min for 3 min. Absorbance at 625 nm was measured to determine the concentration of bacteria suspension.

siRNA transfection

Two hours before transfection, confluent cells were cultured in medium without FBS and antibiotics at 37°C. Subsequently, HMGN2-specific small interfering RNA (siRNA-HMGN2) and negative control RNA (siRNA-NC, RiboBio Ghuangzhou, China) were transfected at a concentration of 10 µmol/L using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 4-6 hours, the transfection complex was replaced with RPMI 1640 medium with 10% FBS and antibiotics, and cells were continuously cultured overnight at 37°C.

Bacterial adherence and invasion assay

T24 and 5637 BECs were seeded at 1×10^5 cells per well into a 24-well plate and allowed to adhere overnight. Then, siRNA transfected cells were washed with PBS and infected with UPEC J96 at a multiplicity of infection (MOI) of 100:1 for 2 h at 37°C. To determine the total number of attached bacteria, cells were washed with warm PBS to remove the non-adherent bacteria, and lysed with 0.25% Triton X-100 in PBS. Cells were then plated on LB agar plates in different dilutions and incubated overnight at 37°C to enumerate colony-forming units (CFU) representing total attached bacteria. To assess the number of internalized bacteria, extracellular bacteria were killed by incubation with a RPMI 1640 medium containing 100 µg/ml of gentamicin at 37°C for additional 1 h prior to Triton X-100 lysis for CFU enumeration as mentioned above.

To measure the roles of various signaling molecules on bacterial invasion, 5637 cells were pretreated with 70 µM NSC23766 (Selleck, Shanghai, China), 20 ng/ml EGF (Cell Signaling Technology, Danvers, USA), 400 ng/ml anisomycin (TCI, Shanghai, China), 20 µM SB203580, 30 µM PD98059, and 100 nM okadaic acid (Beyotime, Haimen, China), respectively, for the indicated time prior to bacterial infection. And these drugs at mentioned concentrations did not affect the viability of 5637 cells.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA from siRNA transfected 5637 cells was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of RNA were measured by NanoDrop™ 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) was used for cDNA synthesis. The synthesized cDNA was served as the template for RT-qPCR amplification of α3 Integrin and β1 Integrin, which was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with TaKaRa SYBR® Premix Ex Taq™ (Takara, Dalian, China). The following primers (Anne-Marie C Dingemans et al., 2010) were used: α3 Integrin forward primer, 5'-AAG GGA CCT TCA GGT GCA-3'; α3 Integrin reverse primer, 5'-TGT AGC CGG TGA TTT ACC AT-3'; β1 Integrin forward primer, 5'-GAA GGG TTG CCC TCC AGA-3'; β1 Integrin reverse primer, 5'-GCT TGA GCT TCT CTG CTG TT-3'. The relative changes of α3/β1 Integrins gene expression in different groups were measured by $2^{-\Delta\Delta ct}$ method.

Western blot

Following treatment, 5637 BECs were rinsed with cold PBS, and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors as well as PMSF (KeyGen BioTech, Nanjing, China). The protein concentration was determined with a BCA Protein Assay kit (Pierce, Rockford, IL, USA). For Western blot analysis, 30 µg total protein from each sample was mixed with sodium dodecyl sulfate (SDS) buffer, and separated by 15% SDS-PAGE gel. Separated proteins were then electrophoretically transferred onto a polyvinylidene difluoride membranes. The membrane was blocked with 5% milk in Tris-buffer saline Tween 20 solution (TBST) for 1 h, and then incubated overnight at 4°C with specific primary antibodies (1:1000 dilution) raised against F-actin (Abcam, Cambridge, UK), Rac1-GTPase (NewEast, Malvern, USA), phospho-FAK, total-FAK, phospho-Src, total-Src (Cell Signaling Technology, Danvers, USA), phospho-p38 MAPK, total-p38 MAPK, phospho-ERK1/2, total-ERK1/2, phospho-JNK, total-JNK (Beyotime, Haimen China). The washed membrane was subsequently incubated with the horseradish peroxidase-conjugated secondary antibody (Beyotime, 1:1000 dilution) for 1 h at room temperature. Signals were detected by enhanced chemiluminescence (MerckMillipore, Bedford, MA, USA) and exposed on ChemiDoc™ MP imager (Bio-Rad, USA). The intensity of each signal was determined relative to corresponding GAPDH bands by Image J.

Flow cytometry analysis

The surface expression of α3/β1 integrins on 5637 cells were quantified by flow cytometry. Briefly, transfected 5637 cells were washed by cold PBS trice, and then fixed with 4% paraf ormaldehyde, permeabilized with 0.1% Triton X-100. Subsequently, cells were incubated in PBS/2% FBS with specific primary rabbit monoclonal antibodies (1:50 dilution) raised against α3/β1 integrins (Abcam, Cambridge, UK) for 1 h at 4°C, and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody for 1 h at 4°C. After several washes, samples were assayed by BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, USA). For F-actin assay, cells were stained with rhodamine-conjugated phalloidin (5 µg/ml) for 1 h at room temperature, and then washed and resuspended in PBS for flow cytometry analysis.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of individual values from three independent experiments. The one-way ANOVA was used to calculate the differences by SPSS 17.0 software. $P < 0.05$ was considered as statistically significant.

Results

HMGN2 knockdown increases UPEC invasion in BECs

Firstly, HMGN2-specific siRNA was used to knockdown the expression of HMGN2, and siR-

Figure 1. Representative RT-qPCR (A) and western blot (B) results showing that HMGN2 mRNA and protein levels were reduced by over 90% and 70% after HMGN2-specific siRNA treatment, respectively.
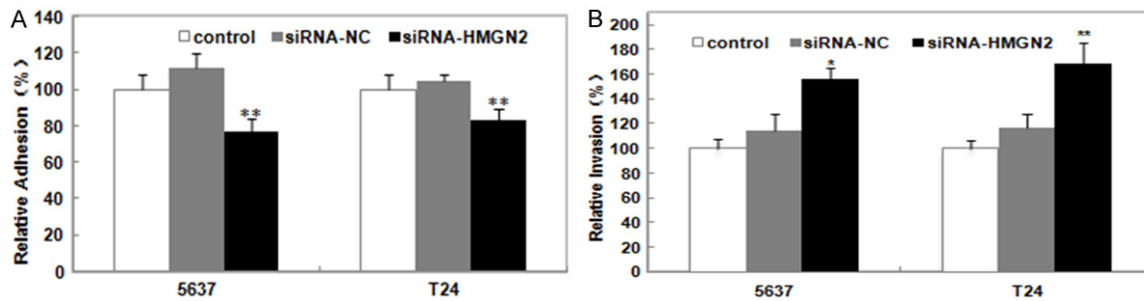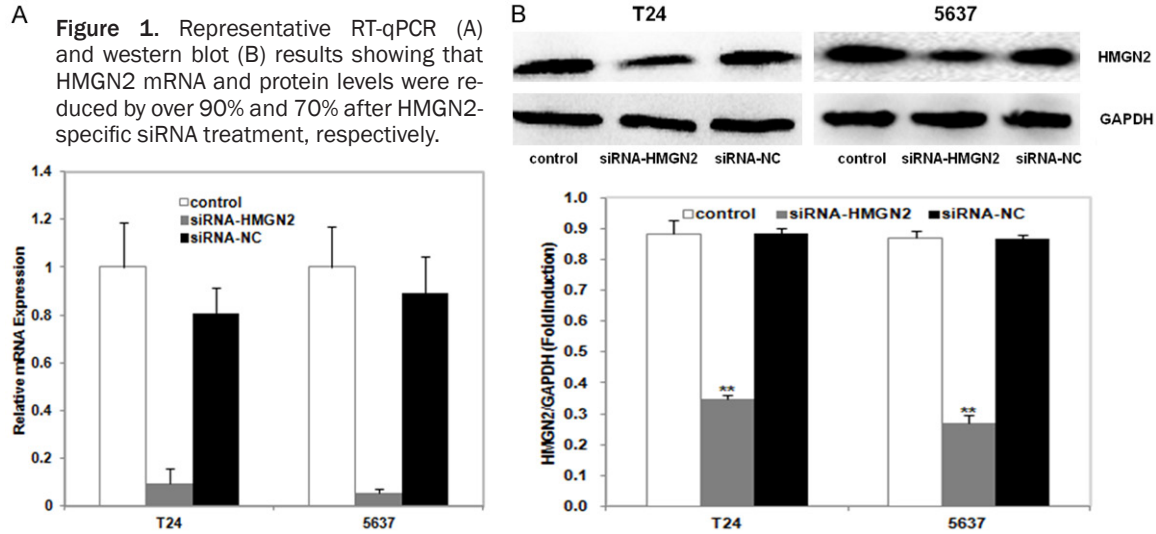

Figure 2. 5637 and T24 BECs were transfected with siRNA-HMGN2 and siRNA-NC for 24 h, then cells were infected with UPEC J96 at an MOI of 100:1 for 2 h. A. BECs were washed and lysed to enumerate adhered UPEC populations via plate count method. B. After incubation BECs were treated with gentamicin and lysed to enumerate invaded UPEC. This study was repeated at least thrice. *P<0.05, **P<0.01 vs. control.

NA-NC was used as a negative control to minimize the effects of RNA. Adhering to and invading the uroepithelial cells is the first step for establishing a successful UPEC infection [17]. To evaluate whether knockdown of HMGN2 affect adhesion and invasion of UPEC in BECs, we infected 5637 and T24 cells with UPEC in the presence or absence of HMGN2-specific siRNA or siRNA-NC. As shown in **Figure 1A** and **1B**, HMGN2-specific siRNA silenced the mRNA and protein expressions of HMGN2 in 5637 cells, respectively. We observed significant decrease in UPEC adhesion in HMGN2 depleted 5637 and T24 cells (**Figure 2A**). In contrast, we discovered that the numbers of UPEC invasion into 5637 and T24 cells were markedly increased in cells previously transfected with HMGN2-specific siRNA (**Figure 2B**). Taken together, knockdown of HMGN2 decreases the UPEC adherence but increases UPEC invasion to BECs.

HMGN2 knockdown enhances host actin polymerization

Host actin cytoskeleton remodeling is a necessary step for bacterial invasion [18]. Meanwhile, HMGN2 has been demonstrated to associate with cytoskeleton remodeling during infection [13, 16]. To test whether HMGN2 knockdown promote host actin polymerization thereby increasing UPEC invasion, flow cytometry was used to measure the polymerization of actin. Interestingly, HMGN2 gene knockdown results in elevated fluorescence intensity of F-actin, the polymerized form of actin, in either UPEC J96 infected or without infected 5637 BECs (**Figure 3A**). HMGN2 knockdown induces actin polymerization were also evaluated using Western blot assay. As shown in **Figure 3B**, HMGN2 gene silencing could increase the protein levels of F-actin in both UPEC infected and uninfected 5637 BECs, thereby suggesting the promotion
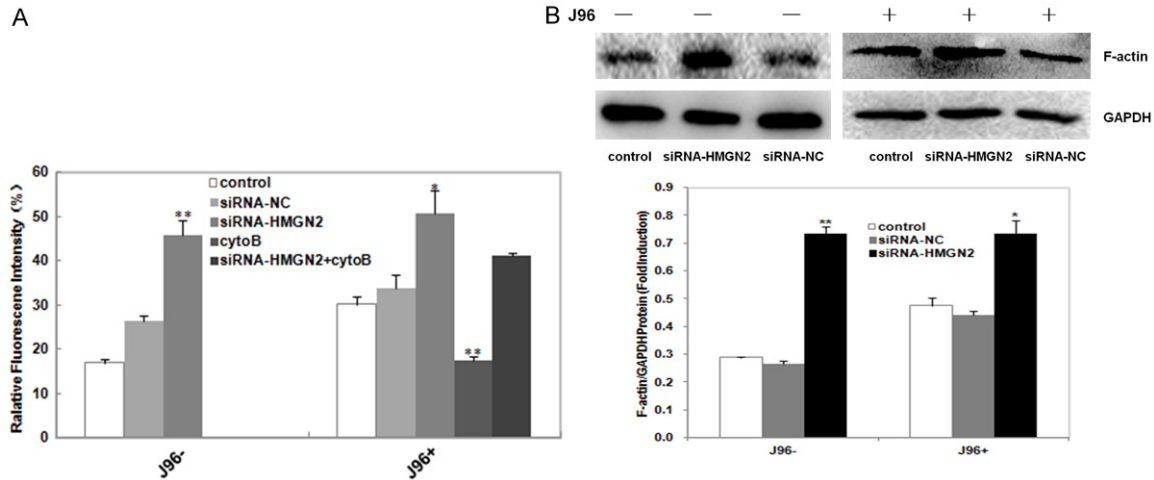
Figure 3. A. 5637 BECs were transfected with siRNA-HMGN2 and siRNA-NC for 24 h, and then infected with UPEC J96 for 2 h. After washing with PBS, cells were stained with rhodamine-conjugated phalloidin. Finally, levels of polymerized actin (F-actin) were measured by flow cytometry. Mean fluorescence intensity was shown in each group. Each column represents the average of at least three results in duplicate. *P<0.05, **P<0.01 vs. control. B. After siRNA transfection and UPEC infection, total cellular proteins were extracted and determined by western blot analysis using antibodies against F-actin and GAPDH as an internal control. Data represent one out of three separate experiments. *P<0.05, **P<0.01 vs. control.
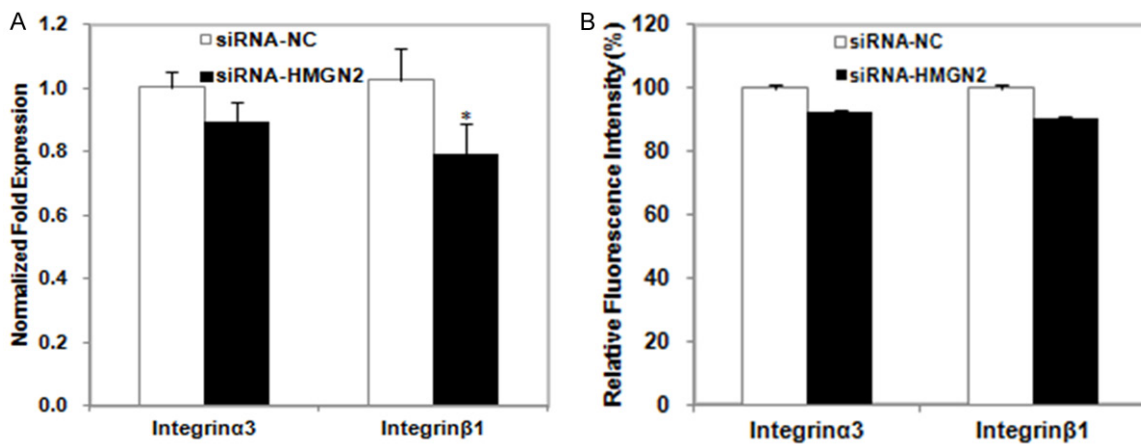

Figure 4. A. 5637 BECs were transfected with siRNA-HMGN2 and siRNA-NC for 24 h, and then infected with UPEC J96 for 2 h. Total RNA was extracted using TRIzol reagent. Gene expressions of integrin α3 and integrin β1 were assessed using RT-qPCR after RNA isolation and reverse transcription. Data represent one out of three separate experiments. *P<0.05, **P<0.01 vs. control. B. Transfected 5637 cells were washed by cold PBS trice. After fixing and transparent, cells were incubated with primary antibodies raised against integrin α3 or integrin β1. Then, cells were incubated with FITC-conjugated secondary antibody. The fluorescence intensities reflect levels of surface-expressed integrin α3 or integrin β1. Mean fluorescence intensity was shown in each group. Each column represents the average of at least three results in duplicate.

of host actin polymerization, which may explain the findings obtained from bacterial adhesion and invasion assays.

HMGN2 gene silencing has no influence on α3/β1 integrins

A plurality of cell surface receptors that drive host actin cytoskeleton remodeling are involved in UPEC adhesion and invasion in BECs, especially the α3/β1 integrins [4]. To gain insight into whether α3/β1 integrins involve in mediated changes in UPEC adhesion and invasion, we assayed the mRNA and protein expressions of α3/β1 integrins via RT-qPCR and flow cytometry, respectively. The RT-qPCR results showed that HMGN2 knockdown did not affect the mRNA expression of α3 integrin, but decreased
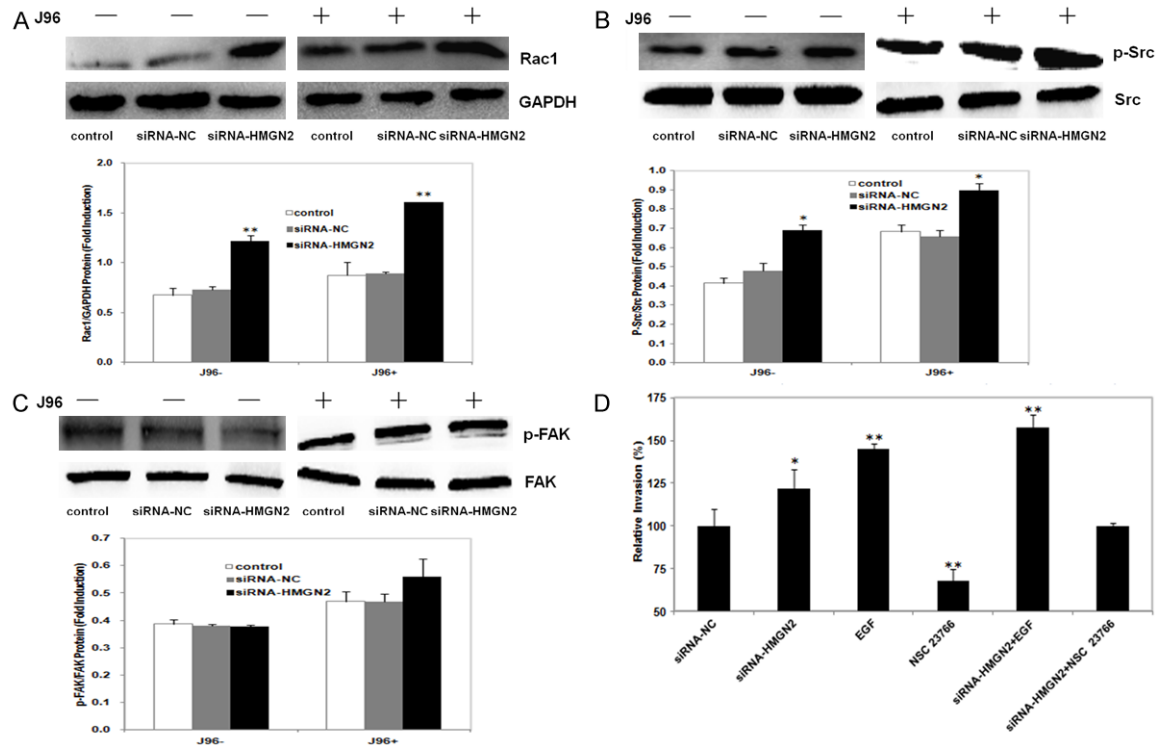
Figure 5. SiRNA-HMGN2 and siRNA-NC transfected 5637 BECs were infected with or without UPEC J96 for 2 h. The proteins were isolated and immunoblotted using anti-Rac1-GTPase, p-FAK, and p-Src, as well as FAK and Src antibodies. An anti-GAPDH antibody was used as a loading control. (A) Knockdown of HMGN2 increased the protein level of Rac1-GTPase. (B) Src phosphorylation level but not (C) FAK phosphorylation level was moderately up-regulated by siRNA-HMGN2 in 5637 BECs. (D) Effects of Rac1-GTPase inhibitor (NSC23766) and agonist (epidermal growth factor, EGF) on the internalization of UPEC J96 into human 5637 BECs. SiRNA-HMGN2 and siRNA-NC transfected cells were treated with NSC23766 (70 μmol) and EGF (20 ng/ml) for 120 min and 30 min, respectively, followed by UPEC J96 infection. The inhibitor NSC 23766 70 μmol, the agonist EGF 20 ng/ml were added 2 h and 30 minutes before the addition of bacteria. The relative UPEC invasion was assessed by colony counts. Each column represents the average of at least three results in duplicate. *P<0.05 vs. control. **P<0.01 vs. control.

the expression of β1 integrin mRNA (**Figure 4A**). Interestingly, no influence on the protein expressions of α3/β1 integrins was observed following HMGN2 knockdown via flow cytometry (**Figure 4B**). Taken together, these results demonstrated that HGMN2 gene knockdown may have little or insignificant influence on the expressions of α3/β1 integrins.

HMGN2 gene silencing induces elevated expression of Rac1-GTPase

In addition to these membrane receptors, some α3/β1 integrins associated signaling factors, including FAK/Src and Rac1-GTPase, are also involved in bacterial invasion and actin cytoskeleton rearrangement [18]. We then examined whether HMGN2 gene silencing could affect the activities of these abovementioned signaling factors. The western blot results showed that HMGN2 knockdown obviously in-

creased the expression of Rac1-GTPase in UPEC uninfected and infected 5637 BECs (**Figure 5A**). Although HMGN2 knockdown resulted in an elevation of phosphorylated Src in uninfected BECs, only a moderate elevation of phosphorylated Src was observed in HMGN2 gene silent 5637 BECs during UPEC infection (**Figure 5B**). Furthermore, HMGN2 knockdown did not affect the phosphorylation of FAK (**Figure 5C**). Therefore, subsequent study focused on the Rac1-GTPase.

To gain insight into the role of Rac1-GTPase in HMGN2 knockdown-mediated promotion of actin polymerization and UPEC invasion, Rac1-GTPase activator human epidermal growth factor (EGF) and inhibitor NSC23766 were used. HMGN2 knockdown mimicked the role of EGF pretreatment, resulted in promotion of UPEC invasion (**Figure 5D**). Otherwise, a significant reduction of UPEC invasion (**Figure 5D**) was
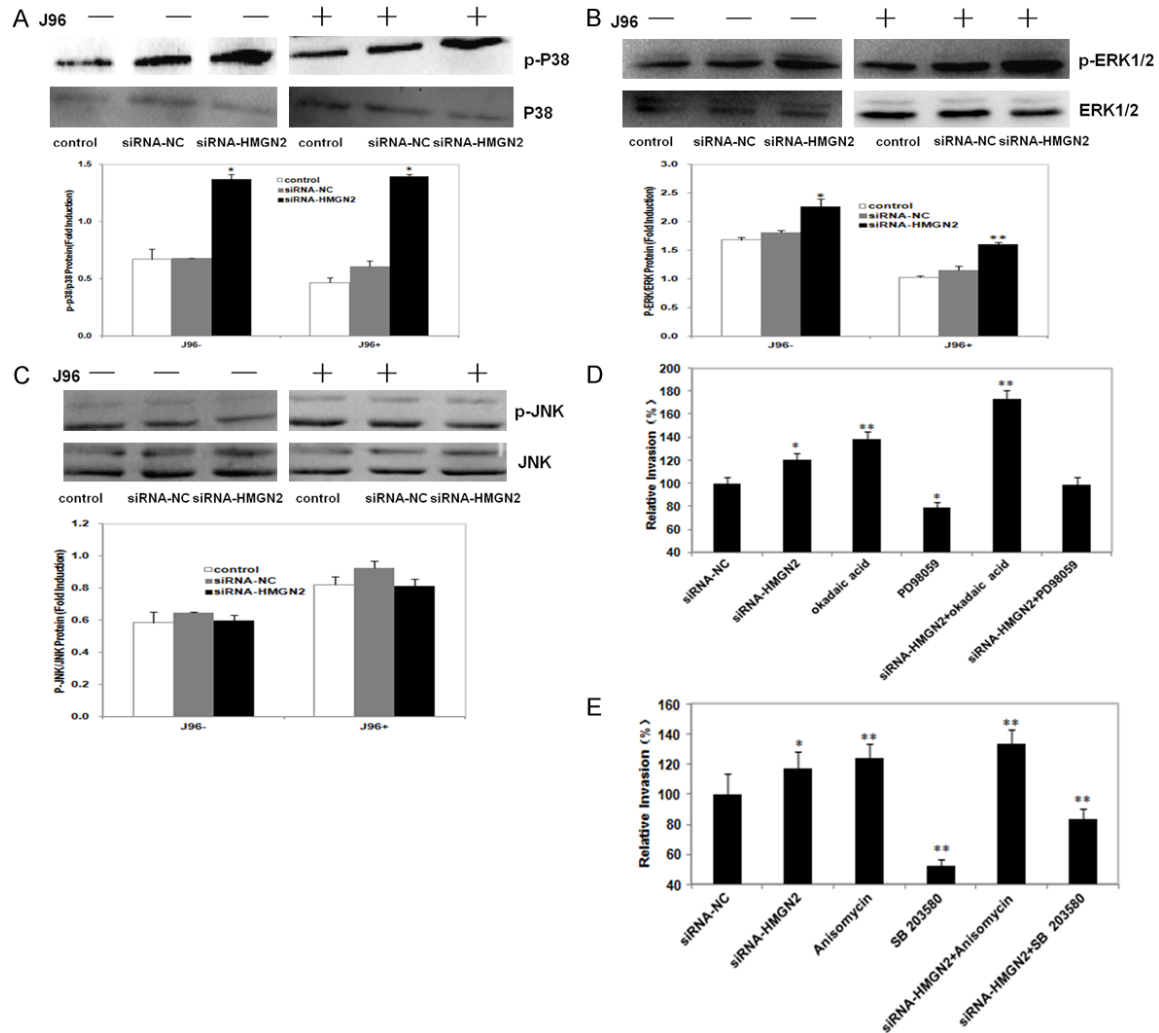
Figure 6. SiRNA-HMGN2 and siRNA-NC transfected 5637 BECs were infected with or without UPEC J96 for 2 h. Equal amounts of total cell lysates were subjected to SDS-PAGE. Proteins were immunoblotted using the indicated antibodies. Western blot results showed that HMGN2 knockdown result in elevated phosphorylation levels of ERK1/2 (A) and p38 MAPK (B), but not JNK (C). (D, E) Effects of ERK1/2 phosphorylation inhibitor (PD98059) and agonist (okadaic acid) as well as p38 MAPK phosphorylation inhibitor (SB203580) and agonist (Anisomycin) on the internalization of UPEC J96 into 5637 cells. SiRNA-HMGN2 and siRNA-NC transfected cells were treated with PD98059 (30 μM) and okadaic acid (100 nM) for 120 min, and SB203580 (20 μM) and anisomycin (400 ng/ml) for 4 h, respectively. Then, cells were infected with UPEC J96 for 2 h. Internalized UPEC was assessed by colony counts. Each column represents the average of at least three results in duplicate. *P<0.05 vs. control. **P<0.01 vs. control.

observed in NSC23766 pretreated 5637 cells. Meanwhile, NSC23766 pretreatment abolished HMGN2 knockdown-mediated promotion of UPEC invasion (**Figure 5D**). These results suggest that HMGN2 knockdown enhances actin polymerization mainly through increasing the expression of Rac1-GTPase, thereby leading to elevation of UPEC invasion.

HMGN2 knockdown increases phosphorylation of p38 MAPK and ERK1/2

As the downstream modulators of Rac1-GT-Pase, MAPKs superfamily proteins including

p38 MAPK, ERK1/2 and JNK are well known to regulate actin cytoskeleton dynamics [6]. Our previous microarray results indicate that HMGN2 gene knockdown influences the MAPKs [13]. Thence, we next analyzed whether MAPKs act as a possible mechanism by which HMGN2 knockdown modulated actin polymerization and UPEC invasion.

As expected, HMGN2 knockdown enhanced phosphorylation levels of p38 MAPK (**Figure 6A**) and ERK1/2 (**Figure 6B**) in both UPEC infected and uninfected 5637 BECs, but did not influence the phosphorylation level of JNK

(**Figure 6C**). These data suggested that p38 MAPK and ERK1/2 might be involved in HMGN2 knockdown mediated promotion of actin polymerization and UPEC invasion. To further probe the potentials of p38 MAPK and ERK1/2 in HMGN2 silencing mediated process, p38 MAPK activator anisomycin and p38 MAPK inhibitor SB203580, as well as ERK1/2 activator okadaic acid and ERK1/2 inhibitor PD98059 were used, respectively. Anisomycin activates p38 MAPK [19], and okadaic acid inhibits the PP2A-mediated dephosphorylation of ERK1/2 thereby leading to elevated ERK1/2 activity [20], both resulted in obvious elevations of UPEC invasion (**Figure 6D** and **6E**). Meanwhile, HMGN2 silencing also mimicked the roles of anisomycin and okadaic acid (**Figure 6D** and **6E**). While, ERK1/2 inhibitor PD98059 abolished the majority of HMGN2 knockdown-mediated promotion of UPEC invasion (**Figure 6D**), and p38 MAPK inhibitor SB203580 neutralized a portion of HMGN2 knockdown-mediated effects (**Figure 6E**). These results suggest that knockdown of HMGN2 caused promotion of actin polymerization and UPEC invasion are mainly mediated by ERK1/2, and partly mediated by p38 MAPK.

Discussion

Our previous results have indicated that exogenous recombinant human HMGN2 can reduce the internalization of *K. pneumoniae* into T24 BECs [16]. Here, we report that endogenous HMGN2 is also involved in the internalization of UPEC into BECs. We found that knockdown of HMGN2 resulted in elevated UPEC invasion into T24 and 5637 BECs through promotion of actin polymerization by increasing the expression of Rac1-GTPase and phosphorylation of p38 MAPK and ERK1/2.

The urinary tract is one of the most intractable mucosal surfaces for microbial colonization. Bacterial adhesion and invasion are the most important strategies for UPEC to evade host innate immune responses and antibiotic therapy, enabling the pathogens to more effectively colonize the urinary tract and establish UPEC infection [21]. In this study, a reduction of bacterial adhesion was observed in HMGN2 knockdown 5637 and T24 BECs. On the contrary, HMGN2 knockdown resulted in an elevated bacterial invasion in BECs (**Figure 2B**). Generally, adherence of bacteria to host mucosal surfaces contributes to bacteria invasion into

host cells [22]. However, bacterial adhesion and invasion are not only closely linked but also relatively independent complex process. And our results indicated that the elevated UPEC invasion induced by HMGN2 gene knockdown in BECs is not relevant to UPEC adhesion. Additionally, multiple factors including the binding of bacteria to host cell membrane receptors, and intracellular signaling cascades as well as their modulated host actin cytoskeleton dynamics have been demonstrated to be involved in the internalization of bacteria [18]. Meanwhile, our previous microarray analysis suggested that depletion of HMGN2 protein altered the expression levels of several functional genes which are associated with intercellular adhesion, and cytoskeletal rearrangements. Hence, this study was focused on these mentioned factors.

As we know, UPEC have developed sophisticated strategies to manipulate the host signals and drive localized host actin rearrangements, which is essential for UPEC invasion [18]. In the present study, we found that depletion of HMGN2 protein markedly promoted the host actin polymerization in both uninfected and UPEC infected 5637 BECs (**Figure 3**), which might contribute to the increase of UPEC invasion. Meanwhile, cytochalasin B, a well known inhibitor of actin polymerization, significantly attenuated both UPEC invasion and HMGN2 knockdown-induced actin polymerization. Therefore, HMGN2 knockdown-mediated elevated UPEC invasion may be mainly dependent on accumulation of F-actin.

Integrins, comprised of α and β subunit heterodimers, are the major and best-characterized trans-membrane receptors, mediate dynamic interactions between the extracellular matrix proteins and the actin cytoskeleton [23]. Many pathogens, such as UPEC UTI89, gain entry into target host cells by binding to α3/β1 integrins either directly or indirectly via matrix proteins [4]. However, the flow cytometric analysis showed that depletion of HMGN2 protein did not affect the surface expression levels of α3/β1 integrins indicating that HMGN2 knockdown induced elevated in actin polymerization and UPEC invasion may not depend on α3/β1 integrins.

F-actin dynamics are modulated downstream of integrins clustering by the recruitment and/or activation of a number of adaptor proteins

[24]. FAK/Src, as the integrin adaptor protein could bridge a number of downstream signaling, and their phosphorylated forms have been shown to alter actin cytoskeletal dynamics in urothelial cells [5, 25]. However, we found that HMGN2 knockdown did not affect the phosphorylation level of FAK, and only caused a moderate increase in phosphorylated Src during UPEC infection (**Figure 5B** and **5C**). These results indicate that actin polymerization and UPEC invasion are dominated by other factors rather than phosphorylation levels of FAK/Src in HMGN2 gene knockdown BECs during UPEC infection.

Similar to our previous microarray results, this study further presents that the host molecule Rac1-GTPase is involved in UPEC epithelial invasion after HMGN2 knockdown. Rac1-GTPase, a member of host Rho GTPases, acts as molecular switches that cycle between GTP-bound (active) and GDP-bound (inactive) form. Once activated, Rac1-GTPase binds to a variety of effectors, including protein kinases and some actin-binding proteins, which directly or indirectly affect the local assembly or disassembly of filamentous F-actin as well as its-mediated phagocytosis, cell deformation and migration [26]. During infection, Rac1-GTPase is critical and necessary because it enhances accumulation of actin filaments at the sites of bacterial entry [27]. In this study, we found significant increased Rac1-GTPase protein expressions in UPEC uninfected and infected BECs that undergo HMGN2 gene knockdown. These data is consistent with our previous microarray data [13]. Furthermore, this result was further confirmed by the observation that HMGN2 knockdown-mediated the majority of UPEC invasion were abolished by inhibitor of Rac1-GTPase NSC23766. In short, these data suggest that depletion of HMGN2 protein induced actin polymerization and UPEC invasion are mainly dependent on the Rac1-GTPase.

As mentioned previously, Rac1-GTPase is critical for immune-receptors mediated phagocytosis in innate immune cells, such as macrophages and epithelial cells, and possesses the ability to activate MAPKs pathways [28]. As the downstream effectors of Rac1-GTPase, MAPKs superfamily proteins including p38 MAPK, ERK1/2 and JNK, modulate the phosphorylation levels of actin-binding proteins including cofilin and profilin, which ensures host cytoskeletal actin polymerization [6]. In addition, our previous study also indicated that host actin polymerization and bacterial invasion are mediated by the activation of ERK1/2 in T24 BECs [16]. Hererin, depletion of HMGN2 protein induced remarkable increased phosphorylated ERK1/2 and p38 MAPK in UPEC infected and uninfected BECs (**Figure 6A** and **6B**), respectively. However, the expression of phosphorylated JNK was not observed in HMGN2 knockdown BECs (**Figure 6C**). Meanwhile, activators of ERK1/2 and p38 MAPK also mimicked the effects of HMGN2 knockdown on host bacterial invasion (**Figure 6D** and **6E**). On the contrary, inhibitor of ERK1/2 PD98059 mostly and inhibitor of p38 MAPK SB203580 partially attenuated HMGN2 knockdown-mediated promotion of bacterial invasion (**Figure 6D** and **6E**), respectively. These findings emphasize the roles of ERK1/2 and p38 MAPK in HMGN2 knockdown-mediated modulatory pathway, helping explain the ability of HMGN2 in UPEC invasion.

In summary, we demonstrate that inhibition of HMGN2 expression in BECs with HMGN2-specific siRNA may promote the actin polymerization and UPEC invasion through increasing the expression of Rac1-GTPase, thereby enhancing the phosphorylated levels of ERK1/2 and p38 MAPK, ultimately. This finding revealed a critical role of HMGN2 in mucosal innate immune response during UPEC infection.

Acknowledgements

Disclosure of conflict of interest

None.

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