Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Antibacterial mechanism of high-mobility group nucleosomal-binding domain 2 on the Gram-negative bacteria *Escherichia coli*[*]

Heng LI[1,2], Xiao-fei SHEN[1,2], Xin-e ZHOU[2], Yan-e SHI[2], Lu-xia DENG[2], Yi MA[2], Xiao-ying WANG[2], Jing-yu LI[†‡2], Ning HUANG[†‡1,2,3]

([1]*Research Unit of Infection and Immunity, Sichuan University, Chengdu 610041, China*)

([2]*Department of Pathophysiology, West China College of Basic and Forensic Medicine, Sichuan University, Chengdu 610041, China*)

([3]*Sichuan University "985 Project Science and Technology Innovation Platform for Novel Drug Development", Sichuan University, Chengdu 610041, China*)

[†]E-mail: xiaohuer1000@126.com; Ning_Huang741@163.com

Received Mar. 29, 2016; Revision accepted June 23, 2016; Crosschecked Apr. 19, 2017

Abstract: Objective: To investigate the antibacterial mechanism of high-mobility group nucleosomal-binding domain 2 (HMGN2) on *Escherichia coli* K12, focusing on the antibacterial and antibiofilm formation effects. Its chemotactic activity on human neutrophils was also investigated. Methods: Human tissue-derived HMGN2 (tHMGN2) was extracted from fresh uterus fiber cystadenoma and purified by HP1100 reversed-phase high-performance liquid chromatography (RP-HPLC). Recombinant human HMGN2 (rHMGN2) was generated in *E. coli* DE3 carrying PET-32a-c(+)-HMGN2. Antibacterial activity of HMGN2 was determined using an agarose diffusion assay and minimum inhibitory concentration (MIC) of HMGN2 was determined by the microdilution broth method. Bacterial membrane permeability assay and DNA binding assay were performed. The antibiofilm effect of HMGN2 was investigated using a crystal violet assay and electron microscopy scanning. The activating effect and chemotactic activity of HMGN2 on neutrophils were determined using a nitroblue tetrazolium (NBT) reduction assay and Transwell chamber cell migration assay, respectively. Results: HMGN2 showed a relatively high potency against Gram-negative bacteria *E. coli* and the MIC of HMGN2 was 16.25 μg/ml. Elevated bacterial membrane permeability was observed in HMGN2-treated *E. coli* K12. HMGN2 could also bind the bacterial plasmid and genomic DNA in a dose-dependent manner. The antibiofilm effect of HMGN2 on *E. coli* K12 was confirmed by crystal violet staining and scanning electron microscopy. However, the activating effects and chemotactic effects of HMGN2 on human neutrophils were not observed. Conclusions: As an antimicrobial peptide (AMP), HMGN2 possessed a good capacity for antibacterial and antibiofilm activities on *E. coli* K12. This capacity might be associated with disruption of the bacterial membrane and combination of DNA, which might affect the growth and viability of *E. coli*.

Key words: High-mobility group nucleosomal-binding domain 2 (HMGN2); Bioactivity; Membrane permeability; Biofilm; Chemotactic activity

http://dx.doi.org/10.1631/jzus.B1600139 **CLC number:** R392.11

1 Introduction

Antimicrobial peptides (AMPs) are produced by a wide variety of organisms in order to protect themselves from infection (Zanetti, 2004; Zhang *et al*., 2015). They often possess a broad-spectrum, non-specific activity against bacteria, viruses, fungi, etc. Primary mechanisms involved in the antimicrobial

‡ Corresponding authors

* Project supported by the National Natural Science Foundation of China (Nos. 30470763, 81470931, and 31401188), the China Medical Board of New York (No. 98-861), and the Youth Foundation of Sichuan University (No. 2014SCU11042), China

ORCID: Heng LI, http://orcid.org/0000-0002-1293-7744

properties of AMPs are inhibiting cell-wall synthesis, altering cytoplasmic membrane, activating the autolysin, inhibiting nucleic acid and protein synthesis, and suppressing enzymatic activity (Brogden, 2005), which differ from that of currently available therapeutic antibiotics, making them non-susceptible to the conventional multidrug resistance mechanism. In addition, some AMPs possess multiple immunoregulatory functions which are manifested as altering mammalian cell membrane properties or binding to its receptors to affect various cellular processes (Lai and Gallo, 2009). Additionally, it has been proved that the secondary metabolites of AMPs are free amino acids which result in fewer side effects than conventional antibiotics. For these reasons, AMPs are promising as novel natural antibiotics.

High-mobility group nucleosomal-binding domain 2 (HMGN2), a member of HMG proteins' superfamily, is defined as an AMP by Reddy *et al.* (2004). It is a non-histone nuclear protein that binds nucleosomes and regulates chromosome architecture and gene transcription, playing a vital role in processes of embryonic development and tumorigenesis (Reeves, 2010). Physical and chemical analyses suggest that HMGN2 is a relatively small, cationic, and amphipathic molecule, with an α-helical structure and containing 89 amino acid residues (9.26 kD, <10 kD) (Feng *et al.*, 2005). Our previous studies suggest that HMGN2 positively modulates the nuclear factor κB (NF-κB) signaling in the nucleus, and promotes lipopolysaccharide (LPS)-induced β-defensin expression in human lung epithelial cell A549 and mice (Deng *et al.*, 2011; 2012). In some cases, HMGN2 could be released into the extracellular medium from interleukin 2 (IL-2) stimulated human mononuclear leukocytes (Feng *et al*., 2005). And we also showed that HMGN2 is a novel antimicrobial molecule, having a potent antimicrobial activity against *Escherichia coli* ML-35p, hepatitis B virus in vitro (Feng *et al.*, 2005; 2009). The antimicrobial effects of HMGN2 on *Klebsiella pneumoniae* internalization and invasion in bladder epithelial cells (Wu *et al*., 2011; Cao *et al*., 2011), and in *E. coli* and *Pseudomonas aeruginosa* have been reported (Feng *et al*., 2005). However, the antibacterial mechanism of HMGN2 has not been fully elucidated.

Bacterial biofilms play a critical role in the resistance to antibiotic chemotherapy and human immune defenses (Høiby *et al*., 2010). Therefore, we evaluated the antibacterial and antibiofilm effects and the relative mechanisms of HMGN2 on *E. coli* K12. In addition, based on the multiple biological functions of AMPs, the activating effects and chemotactic activity of HMGN2 on human neutrophils were investigated.

2 Materials and methods

2.1 Bacterial strains and cultures

E. coli (ATCC25922 and K12), *P. aeruginosa* (ATCC27853 and PAO1), *Acinetobacter baumannii* (clinical isolate), *K. pneumoniae* (clinical isolate), *Staphylococcus aureus* (ATCC25923), *Streptococcus faecalis* (clinical isolate), and *Canidia albicans* (ATCC90028) were used in this study. The clinical isolates were obtained from the Department of Pathophysiology in Medical West China Hospital of Sichuan University (Chengdu, China). Bacteria were cultured in a shaking lysogeny broth (LB) at 37 °C. After centrifugation, the bacterial concentration was adjusted in phosphate buffer saline (PBS) and determined by measuring absorbance at 600 nm.

2.2 Human participants and sample collection

This study was subject to approval by the Ethics Committees of Sichuan University (No. JJ2014010) and was performed according to the Helsinki Declaration. All subjects provided informed written consent. Tumor tissue samples were collected during surgery resection from women with uterus fiber cystadenoma (aged from 30–40 years, without antibiotic or hormonal therapy prior to operation) in our hospital. The peripheral blood was collected from healthy adult volunteers (male, 24 years old) with heparin. The neutrophils were separated from the peripheral blood through a human neutrophils separation medium, then washed twice with sterile PBS, and resuspended in Roswell Park Memorial Institute 1640 (RPMI-1640) medium.

2.3 Peptide purification and preparation

Human tissue-derived HMGN2 (tHMGN2) was extracted from fresh uterus fiber cystadenoma, and purified by HP1100 reversed-phase high-performance liquid chromatography (RP-HPLC, Hewlett-Packard, USA). Initially, the tumor tissues were washed with

pre-cold PBS, finely chopped, and ground in liquid nitrogen. The tissues were homogenized in lysis buffer (5% (v/v) perchloric acid) and centrifuged at 4 °C. Supernatants were dialyzed in a 3500 molecular weight cut off tube (Spectrum Medical Industrial Co., USA) against water at 4 °C for 48 h, and then lyophilized and re-dissolved in 0.1% (v/v) trifluoroacetic acid. The peptide was purified by RP-HPLC on a Vydac 218TP C18 (0.46 cm×25 cm, 5 μm, 30 °C) equilibrated with 0.1% trifluoroacetic acid. The solution of 60% acetonitrile/0.1% trifluoroacetic acid was used as the eluant. The flow rate was 1 ml/min. Absorbance at 214 nm was measured, and the major peak in the chromatogram was identified.

Recombinant human HMGN2 (rHMGN2) was generated in *E. coli* DE3 and purified by affinity chromatography and enzyme digestion methods. The transformed *E. coli* DE3 carrying PET-32a-c(+)-HMGN2 was cultured in Luria-Bertani containing 25 μg/ml ampicillin. Then 0.1 mmol/L isopropyl-β-D-thiogalactoside (IPTG) was added to induce protein expression at 30 °C. Bacterial cells were lysed by sonication and centrifuged at 4 °C. Then supernatants were purified using HisTrap™ Kit column (Pharmacia, Piscataway, USA). The eluents were dialyzed, purified by RP-HPLC, and cleaved by thrombin digestion.

Protein concentration was determined using the bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, USA). The purity protein was confirmed by tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE), acid-urea-PAGE (AU-PAGE), and Western blotting with a primary anti-HMGN2 monoclonal antibody (Sigma, St. Louis, USA).

2.4 Agarose radial diffusion antimicrobial assay

Agarose diffusion assay was used to evaluate the antibacterial activity of HMGN2 as previously described (Lehrer *et al.*, 1991). Plates were poured with 15 ml molten agarose gel containing 0.03% (0.3 g/L) soya peptone (Sigma, St. Louis, USA), 1% (0.01 g/ml) low electrolyte agarose (Sigma, St. Louis, USA), and 30 μl of *E. coli* suspension (0.5 McFarland scale, about 1×10^8 colony-forming unit (CFU)/ml). Subsequently, different concentrations of HMGN2 (5 μl) were added to 3-mm-diameter wells, which had been cut using sterile hole punchers and incubated at 37 °C for 3 h. Finally, the plates were overlaid with molten medium (50 °C) containing 1% low electrolyte agarose and 3% (0.03 g/ml) trypticase soybean and incubated at 37 °C overnight. The diameter of the zone of inhibition around the punched hole was used to assess the antibacterial potential against bacteria. Additionally, PBS was used as the negative control and lysozyme and human neutrophil peptide (HNP) were employed as the positive controls.

2.5 Minimum inhibitory concentration assay

Minimum inhibitory concentrations (MICs) of HMGN2 were determined by the microdilution broth method following the guidelines of the Clinical Laboratory Standards Institute (Wiegand *et al.*, 2008). Mid-logarithmic bacterial cultures were diluted with sterile PBS to a final cell density of 1×10^6 CFU/ml, then 40 μl of the bacterial suspension was seeded in a 96-well plate. A volume of 10 μl of HMGN2 dilution with 10 graded serial concentrations within the 500–1 μg/ml range was added and the plate was incubated at 37 °C for 12 h. The lowest concentration of HMGN2 that can completely suppress the growth of the bacteria was identified as the MIC.

2.6 Bacterial membrane permeability test

A bacterial membrane permeability assay was conducted as described previously (Wang *et al*., 2014). A total of 200 μl bacterial suspension (1×10^8 CFU/ml) was incubated with HMGN2 or bovine serum albumin (BSA, 5 μg/ml) at 37 °C for 240 min. Thereafter, the bacterial suspensions were centrifuged (12000 r/min) at 4 °C for 2 min. The absorbance of supernatants was determined by spectrophotometry at 260 and 280 nm, respectively. An elevated absorbance indicates higher bacterial cell membrane permeability.

2.7 DNA binding assay

E. coli K12 plasmid DNA was extracted using Eazy Nucleic Acid Isolation E.Z.N.A. Plasmid Miniprep Kit (Omega, Georgia, USA) following the manufacturer's protocol. Genomic DNA was extracted by a cetyl triethyl ammonium bromide (CTAB)/phenol-chloroform DNA extraction method (Wang *et al*., 2013). Overnight-cultured *E. coli* K12 was harvested and resuspended in a Tris-ethylene diamine tetraacetic acid (EDTA) buffer (pH 8.0). Then the bacterial suspension was incubated with 20 mg/ml

protease K and 10% SDS at 37 °C for 60 min. The lysate was incubated with 5 mol/L NaCl and CTAB/NaCl solution for 30 min at 65 °C. Proteins were removed by addition of phenol/chloroform (1:1) twice. The supernatant was further precipitated by sodium acetate/alcohol (1:20), rinsed twice with 80% alcohol. The extracted DNA was dissolved in a Tris-EDTA buffer. The absorbances at 260 and 280 nm (A_{260} and A_{280}) were recorded. A_{260}/A_{280} and electrophoresis were used to analyze the DNA purity.

For the DNA binding assay (Park *et al.*, 1998), 0.5 μg genomic DNA and 0.1 μg plasmid DNA were mixed with different amounts of HMGN2, rabbit neutrophil defensin, and BSA (as negative control) in a binding buffer (5% glycerol, 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 20 mmol/L KCl, and 50 μg/ml BSA, 10 mmol/L Tris-HCl, pH=8.0), respectively and were incubated at room temperature for 3 h. Subsequently, 4 μl of native loading buffer (10% Ficoll 400, 50 mmol/L EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol, and 10 mmol/L Tris-HCl, pH=7.5) was added and an aliquot of 12 μl was applied to a 0.7% agarose gel electrophoresis, and finally imaged using the Molecular Image® Gel Dox XR+ gel imager (Bio-Rad, California, USA).

2.8 Crystal violet staining

The effect of HMGN2 on biofilm formation was investigated using a crystal violet assay as previously described (Mitra *et al*., 2013). *E. coli* K12 (2×10^6 CFU/ml) was mixed with 10 ml of tryptic soy broth (TSB) medium. A volume of 200 μl of the suspension was added to each well of a 96-well plate with or without HMGN2 (1× MIC) and the plate was placed in an incubator at 37 °C for 3 or 6 d with a fresh medium change every 24 h. Upon biofilm formation, HMGN2 was added to each well and inoculated for another 2 d. After incubation, plates were then washed twice with PBS to remove the non-adherent bacteria. Adherent bacterial cells were stained with 25 μl of 1% crystal violet for 15 min; excess crystal violet was then rinsed twice with PBS. Subsequently, the dye bound to the adherent bacterial cells was re-dissolved with 200 μl of 95% ethanol. The optical density (OD) of each well was measured at 595 nm.

2.9 Scanning electron microscopy assay

Bacterial incubation and HMGN2 treatment were carried out using the same methods previously described. After HMGN2 treatment, the filter membranes were fixed with 3% glutaraldehyde and 1% osmic acid in 0.2 mol/L PBS for 4 h at 4 °C and dehydrated in a series of ethanol solutions with graded concentration (30%–100%). Thereafter, samples were dried in a critical point dryer, and sputter-coated with gold and imaged using a JSM-7500F electron microscope (JEOL, Japan) at 20 kV.

2.10 Nitroblue tetrazolium reduction assay

The activating effect of HMGN2 on neutrophils was determined using an nitroblue tetrazolium (NBT) reduction assay as described before (Park *et al*., 1968). A total of 5×10^5 neutrophils were incubated in a freshly prepared solution containing PBS, 0.2% NBT (Sigma, Shanghai, China), and 1% human albumin as well as various concentrations of HMGN2 or LPS (Sigma, Shanghai, China) at 37 °C for 30 min. Then, the cells were collected and stained with Wright's dye. Finally, at least 100 cells counted in 6 high power fields per glass slide were observed under the light microscope, and the NBT-positive cells (with blue-black granules of formazan in cytoplasm) were calculated.

2.11 Transwell chamber cell migration assay

Transwell chamber cell migration assay was conducted as has been described before (Degryse *et al.*, 1999). Shortly, Transwell chambers were used with microporous filters (3 μm pore size). The human neutrophils were seeded in the upper well of the Transwell chamber with about 2×10^5 cells/well. The HMGN2 was diluted in a serum-free DMEM medium and was added to the lower well of the Transwell chamber. Thirty minutes later, the filters were rinsed twice with PBS, fixed with methanol, and stained with a solution of Giemsa. Finally, the filters were washed and the migrating cells in 5 fields per filter were observed under a light microscope (×200). Casein was used as a positive control and the serum-free DMEM medium was taken as the negative control.

2.12 Statistical analysis

Data were expressed as mean±standard deviation (SD) of triplicate experiments. The differences among groups were analyzed by one-way analysis of variance (ANOVA) using the SPSS 17.0 software (SPSS Inc., Chicago, USA). Differences with $P<0.05$ were considered to be statistically significant.

3 Results

3.1 Peptide isolation and purification

Typical human tHMGN2 was observed in Figs. 1a–1d. The retention time of the tHMGN2 was about 17.5 min (Fig. 1a). Then this peak was collected, and identified by tricine-SDS-PAGE. The molecular weight of this protein was about 14 kD (Fig. 1b). Meanwhile, the protein was identified as HMGN2 by Western blotting (Fig. 1c). Finally, AU-PAGE assay suggested that HMGN2 was a cationic molecule (Fig. 1d).

Similarly, the rHMGN2 was observed in Figs. 1e–1h. The crude peptide was successively purified by affinity chromatography and RP-HPLC. The major peak was collected and cleaved by thrombin, which was further purified by RP-HPLC. The retention time of rHMGN2 was about 21 min (Fig. 1e). Then this peak was collected, and identified by SDS-PAGE. The molecular weight of this protein was about 14 kD (Fig. 1f). Then, the protein was identified as HMGN2 by Western blotting (Fig. 1g). The AU-PAGE result showed that HMGN2 was an amphipathic molecule (Fig. 1h).

3.2 Antimicrobial effects and MIC of HMGN2

As shown in Table 1, tHMGN2 and rHMGN2 both possessed powerful antimicrobial activity on *E. coli*, and lower potency on *A. baumannii*. However, antimicrobial effects of HMGN2 on *P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *S. faecalis*, and *C. albicans* were not observed in the present study. Both tHMGN2 and rHMGN2 could obviously inhibit the growth of *E. coli* K12 (Fig. 2), and the MIC of HMGN2 against *E. coli* K12 was 16.25 μg/ml as obtained through the broth microdilution method.

3.3 Bacterial cell membrane permeability assay

To evaluate whether HMGN2 disrupted the bacterial membrane integrity, the amounts of bacterial nucleic acids and proteins released from HMGN2 or vehicle-treated bacterial cells were measured at

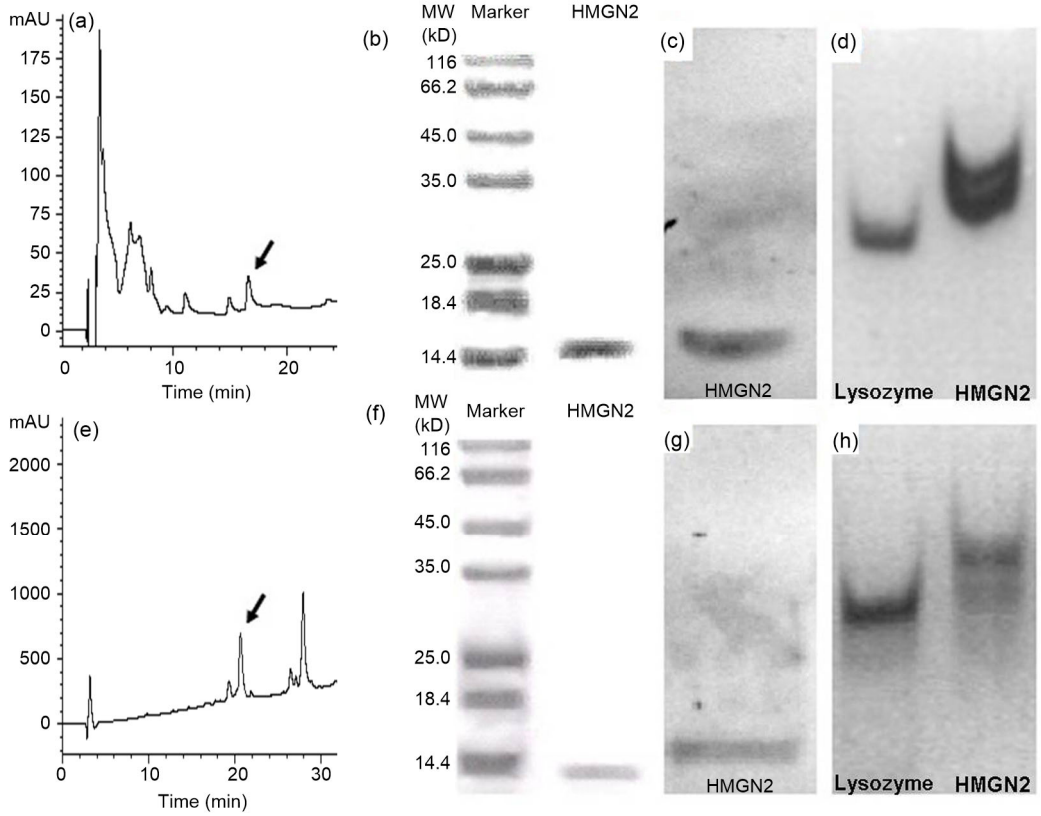

Fig. 1 Isolation and purification of human tissue-derived (a–d) and recombinant (e–h) HMGN2

(a, e) RH-HPLC chromatogram profiles of HMGN2 from the human uterine fibroid cystadenoma and *E. coli* DE3 carrying PET-32a-c(+)-HMGN2; (b, f) Identification of the molecular weight via SDS-PAGE assay; (c, g) Western blotting assay for the identification of HMGN2; (d, h) Identification of molecular charge through AU-PAGE assay. MW: molecular weight

Table 1 Antimicrobial activity analysis of human tissue-derived and recombinant HMGN2

Group	*E. coli*		*P. aeruginosa*		*A. baumannii* clinical isolate	*K. pneumonia* clinical isolate	*S. aureus* 25923	*S. faecalis* clinical isolate	*C. albicans* 90028
	25922	Clinical isolate	27853	POA1					
Vehicle	−	−	−	−	−	−	−	−	−
tHMGN2	+++	+++	ND	ND	+	−	ND	ND	ND
rHMGN2	+++	+++	−	−	+	−	−	−	−
HNP	+++	+++	−	−	+	−	−	−	−

The antimicrobial activities of HMGN2 were expressed as the inhibitory-zone diameters (*d*). +++: $d \geq 8$ mm; ++: 8 mm>*d*>5 mm; +: 5 mm≥*d*≥3 mm; −: no antimicrobial activity, $d < 3$ mm; ND: not done. Vehicle: PBS; tHMGN2: tissue-derived HMGN2; rHMGN2: recombinant HMGN2; HNP: human neutrophil peptide

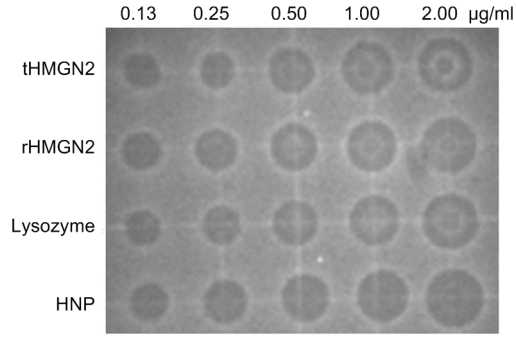

Fig. 2 Inhibition of the growth of *E. coli* K12 by human tissue-derived and recombinant HMGN2

Representative inhibitory zones of HMGN2 for *E. coli* K12 strain in agarose radial diffusion plate

260 and 280 nm by ultraviolet (UV) spectrophotometry. After being incubated with HMGN2 for 4 h, the bacterial membrane permeability significantly increased, which manifested as elevated OD values at 260 and 280 nm (Fig. 3). This result suggests that the antibacterial effect of HMGN2 is involved in bacterial membrane damage.

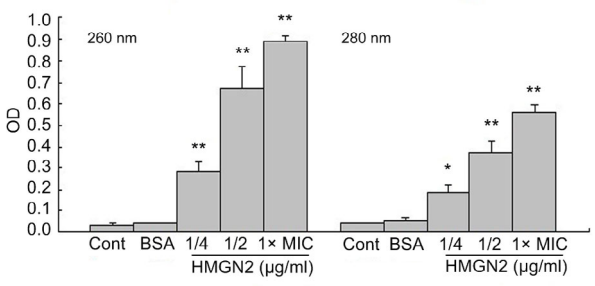

Fig. 3 Bacterial cell membrane permeability change by HMGN2

Bacteria were incubated with PBS (Cont), BSA, and HMGN2 (1/4, 1/2, and 1× MIC) for 4 h at 37 °C. Then the OD values at 260 and 280 nm of supernatants were measured. Data are presented as mean±SD, $n=9$. $^{*}P<0.05$, $^{**}P<0.01$ vs. control

3.4 Binding assay between HMGN2 and bacterial genomic/plasmid DNAs

To further explore the mechanisms of the antibacterial effect, the genomic and plasmid DNAs binding properties of HMGN2 were examined. As shown in Fig. 4, HMGN2 suppressed the migration of bacterial genomic and plasmid DNAs above the weight ratios of 1:10 (Fig. 4a) and 1:8 (Fig. 4b), respectively. In contrast, the rabbit defensin and BSA did not suppress the migration of DNA, which indicated that rabbit defensin and BSA could not bind to the bacterial genomic and plasmid DNAs.

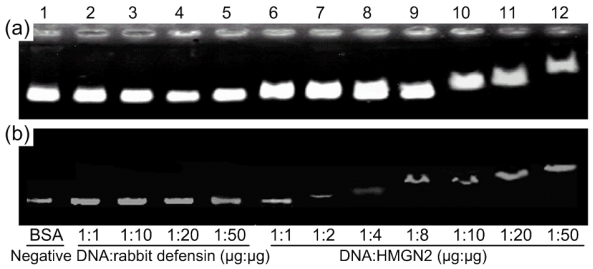

Fig. 4 Binding between HMGN2 and genomic/plasmid DNAs of *E. coli* K12

(a) Genomic DNA; (b) Plasmid DNA. 1: negative control (BSA, 5 μg/ml); 2–5: the ratio of DNA:rabbit defensin (1:1, 1:10, 1:20, 1:50); 6–12: the ratio of DNA:HMGN2 (1:1, 1:2, 1:4, 1:8, 1:10, 1:20, 1:50)

3.5 Disruption of *E. coli* K12 biofilm by HMGN2

Fig. 5 depicts the changes of biofilm formation in HMGN2 (1× MIC)-treated *E. coli* K12 strain. Firstly, crystal violet staining was used to evaluate the effect of HMGN2 on the biofilm of *E. coli* K12. After 3 (Fig. 5a) and 6 d (Fig. 5b) exposure to PBS, BSA, and HMGN2, the K12 strain still produced moderate biofilm. However, only HMGN2 could obviously disrupt the biofilm formation and damage the formed

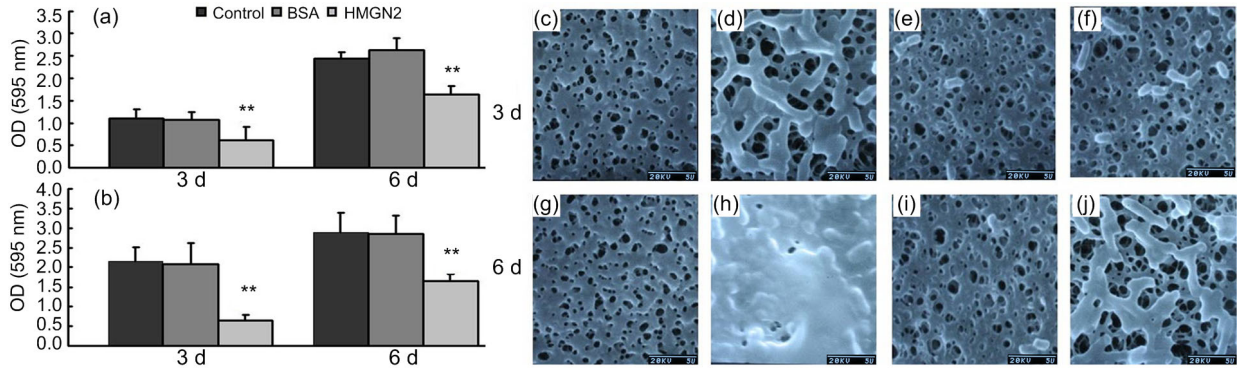

Fig. 5 Disruption of *E. coli* K12 biofilm by HMGN2

(a) Early and mature biofilm staining via crystal violet. (b) Early- and maturely-formed biofilm staining via crystal violet. Data are presented as mean±SD, n=9. * P<0.05, ** P<0.01, vs. control. (c–j) Representative images of scanning electron microscopy, original magnification ×5000. (c, g) Negative control of *E. coli* K12 biofilm; (d) Early biofilm (3 d); (h) Mature biofilm (6 d); (e) Early biofilm formation cultured in the presence of HMGN2; (i) Mature biofilm formation cultured in the presence of HMGN2; (f) Change of early-formed biofilm cultured in the presence of HMGN2; (j) Change of maturely-formed biofilm cultured in the presence of HMGN2

biofilm (Figs. 5c–5j). A similar result was obtained from the scanning electron microscopy observation. The control membrane showed a well-developed biofilm growth of *E. coli* K12 (Figs. 5d and 5h), whereas *E. coli* K12 treated with HMGN2 developed poor biofilm formation (Figs. 5e and 5i) compared to that of the control sample. HMGN2 also could damage the early- and maturely-formed biofilms (Figs. 5f and 5j). Taken together, these results demonstrated that HMGN2 disrupted the biofilm of *E. coli* K12.

3.6 Nitroblue tetrazolium reduction assay and Transwell chamber migration assay

To demonstrate the activating effect and chemotactic activity of HMGN2 on human neutrophils in vitro, the NBT reduction assay and Transwell chamber migration assay were carried out. As shown in Fig. 6a, 100 μg/ml LPS could obviously activate the human neutrophils. However, various concentrations of HMGN2 did not show the ability to induce activation of neutrophils in vitro. The Transwell chamber assay (Figs. 6c–6e) suggested that HMGN2 did not possess chemotactic activity on human neutrophils. Hence, HMGN2 was not considered as a neutrophil activator or chemokine.

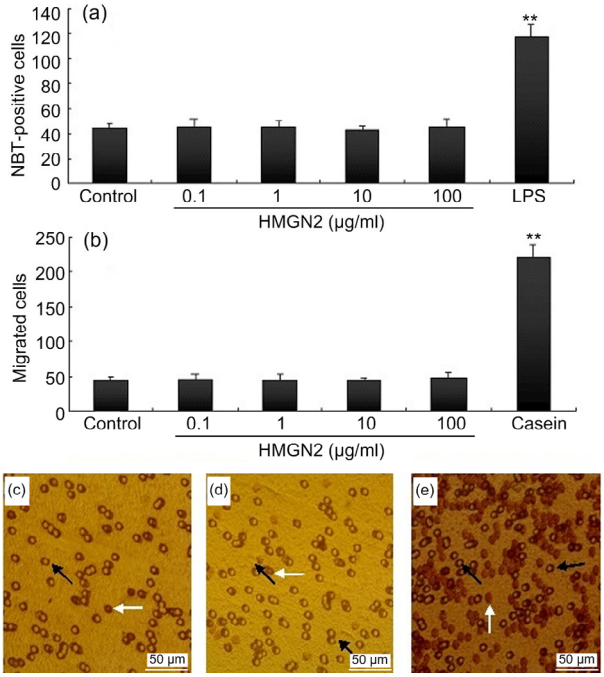

Fig. 6 Nitroblue tetrazolium (NBT) reduction assay (a) and Transwell chamber migration assay (b–e)

(a, b) Data are presented as mean±SD, n=9; (c–e) Representative images of Transwell assay, original magnification ×200. (a) The number of NBT-positive cells; (b) The number of migrated cells; (c) Negative control; (d) 100 μg/ml HMGN2; (e) 5 mg/ml casein (as positive control). White arrow: migrated cells; Black arrow: filter holes

4 Discussion

As the incidence of antibiotic resistance in pathogenic bacteria rises worldwide, there is an urgent need to develop novel antimicrobial agents to fight various types of infectious disease (Stewart and Costerton, 2001). AMPs, so-called “natural antibiotics”, have drawn the focus of pharmacists because of their potential for antibiotic resistance (Brogden, 2005). The present study set out to probe the antibacterial

mechanism of an AMP named HMGN2 on the *E. coli* K12 strain, focusing on the antibacterial and antibiofilm effects, as well as the activating effects and chemotactic effects on human neutrophils in vitro.

In the present study, both tHMGN2 and rHMGN2 showed antibacterial activities against *E. coli* and *A. baumannii*. However, no effects on other Gram-negative bacteria (such as *P. aeruginosa*, *K. pneumoniae*), Gram-positive bacteria (like *S. aureus*, *S. faecalis*), or fungi (like *C. albicans*) were observed. This result indicated that HMGN2 possessed relatively high potency against Gram-negative bacteria *E. coli*. The model organism *E. coli* K12 strain was used to evaluate the antibacterial activity of these two kinds of HMGN2. The result showed that the effectiveness of tHMGN2 and rHMGN2 was almost equivalent (Fig. 2) and the MICs of these two kinds of HMGN2 were 16.25 μg/ml for *E. coli* K12. Hence, rHMGN2 was used in the next assay.

We further investigated the antibacterial mechanisms of HMGN2 on *E. coli*. AMPs possess unique properties and alternative modes of action different from the conventional antibiotics which penetrate into bacterial cells and interfere with intracellular targets (Liu *et al*., 2013). Cationic and amphipathic AMPs that can bind to the surface of bacterial cells are composed of the negatively charged components in a non-specific electrostatic interaction (Brogden, 2005). Then, the AMPs insert into the hydrophobic membrane core, and eventually form transmembrane pores that cause an unrestricted, lethal flux of intracellular ions and other molecules from the cytoplasm (Bolintineanu *et al*., 2010). Therefore, the release of intracellular components including DNA, RNA, and proteins serves as a useful indicator of bacterial membrane integrity (Chen *et al*., 2013). We observed a dose-dependent manner for DNA, RNA, or protein release (Fig. 3) when treated with HMGN2 by testing the absorbance at 260 and 280 nm. Therefore, we can suggest that HMGN2 damaged the cell membrane integrity of the bacteria. HMGN2 is a cationic and amphipathic molecule (Feng *et al*., 2005), which may target the bacterial membrane through binding to the negatively charged membrane components of *E. coli* (such as phosphate groups from LPS). Then the α-helix of HMGN2 inserts into the membrane and induces the membrane pore formation so that the intracellular contents can be released into the extracellular medium, eventually resulting in the death of bacteria. Furthermore, a result from our laboratory showed that HMGN2 inhibited the synthesis of capsular polysaccharide in Gram-negative bacteria *K. pneumoniae* (data not shown). When approaching the bacterial surface, AMPs traverse capsular polysaccharides in order to act on the bacterial membrane. The inhibitory potential of HMGN2 on capsular polysaccharides may contribute to the fact that HMGN2 more easily accesses the cytoplasmic membrane, and acts on the bacterial lipid bilayers.

It is also possible that some AMPs target other different intracellular components, and membrane disruption may not be the only cause of bacterial cell death (Brogden, 2005). Once translocate into cells, AMPs alter cytoplasmic membrane septum formation, inhibit cell-wall/nucleic acid/protein synthesis, or suppress enzymatic activity (Brogden, 2005; Sarda-Mantel *et al*., 2007). For example, a histone-derived AMP named buforin II, which first crosses the cytoplasmic membrane, and then binds to nucleic acids, eventually causes bacterial cell death (Xie *et al*., 2011). Similar to histone, HMGN2 functions as a nuclear factor that can bind to nucleosomes, thereby regulating chromosome architecture and gene transcription in the nucleus (Furusawa and Cherukuri, 2010; Reeves, 2010). Upon release into the extracellular medium, we explored whether HMGN2 exerts similar effects on bacteria to those of the histone-derived AMPs. We found that HMGN2 could bind genomic DNA of *E. coli* K12 and alter the electrophoretic mobility of DNA in 1% agarose gels in a dose-dependent manner (Fig. 4). This result also suggested that the antibacterial activity of HMGN2 on *E. coli* might be associated with its ability to bind the DNA. HMGN2 also can bind to plasmid DNA. Plasmids are responsible for the capture of different antibiotic resistance genes, and for inducing the multidrug resistance phenotype (Hawkey and Jones, 2009). The plasmid DNA binding capacity might imbue HMGN2 with the potential to reduce antibiotic resistance. We assume that the ability of HMGN2 to bind the genomic and plasmid DNAs might disrupt gene transcription and translation, and thereby affect growth and reproduction of bacteria. However, proper experiments on the transcription and translational level should be conducted to explain the antibiotic resistance of HMGN2.

Generally, bacteria adhere to damaged tissue by formation of a biofilm consisting of a polysaccharide, protein, and DNA (Stewart and Costerton, 2001), leading to persistent and chronic infections (Costerton *et al*., 1999) and inherent resistance to antibiotic chemotherapy as well as phagocytosis and other components of the human body's immune defense system (Høiby *et al*., 2010). As is well known, the process of a biofilm consists of five stages: initial attachment, irreversible attachment, microcolony and early biofilm formation, biofilm maturation, and final dispersion (O'Toole *et al*., 2000). The crystal violet staining and scanning electron microscopy showed that the treatment of HMGN2 in the *E. coli* K12 strain reduced the early (3 d) and mature (6 d) biofilm formation (Figs. 5a and 5c–5f). Simultaneously, HMGN2 also could damage the early- and maturely-formed biofilm of *E. coli* (Figs. 5b and 5g–5j). Additionally, HMGN2-treated *E. coli* with impaired biofilms might be more easily recognized by the host immune system or be killed by antibiotics. Therefore, the suppression of biofilm formation by HMGN2 is also conducive to the prevention and treatment of *E. coli*-induced chronic infections.

Recently, a growing body of work suggests that AMPs also act as gene-encoded natural antibiotics (Brogden, 2005). Some AMPs such as defensins and cathelicidins activate a number of responses in host innate immune cells by inducing chemokines and cytokines release, promoting leukocytes recruitment, and activating antigen presenting cells (Steinstraesser *et al.*, 2011). Previous studies indicated that some endogenous mediators that are not traditionally considered as AMPs, such as HMG superfamily protein HMGB1 and HMGN1, act as alarmins, and are also shown to exhibit chemoattracting and activating effects on dendritic cells (Rovere-Querini *et al.*, 2004; Yang *et al*., 2012). Apart from the dendritic cells, neutrophils are other key innate immune cells in the human immune system. They can be rapidly recruited to the site of infection in response to chemokines, and trigger microbicidal mechanisms such as AMP release and rapid reactive oxygen species (ROS) production (Bratton and Henson, 2011). The NBT reduction assay has been used to test ROS production and neutrophil activation (Tunc *et al.*, 2010) and the results showed that 100 μg/ml HMGN2 could not induce the activation of human neutrophils (Fig. 6a). On the other hand, Transwell chamber assay suggested that HMGN2 did not exhibit a chemoattracting effect on neutrophils in vitro (Figs. 6b and 6c). Yang *et al.* (2012) reported that HMGN2 did not exhibit the capacity to induce phenotypic maturation of human monocyte-derived dendritic cells, which also confirms our results. Hence, HMGN2 does not possess an alarmin-like effect and does not exhibit the ability to modulate the host immune function. Although HMGN2 has limited homology (about 50%) with HMGN1 and is only 10 amino acids shorter than HMGN1 (Furusawa and Cherukuri, 2010), it is unclear why HMGN2 does not possess an alarmin-like effect on innate immune cells.

In conclusion, the present study indicates that the HMG superfamily protein HMGN2 possesses a good capacity to have an antibacterial effect on the Gram-negative bacteria *E. coli*. This ability might be associated with damage of the bacterial cell membrane and combination of DNA. HMGN2 also exhibits a good antibiofilm effect on *E. coli*, but a poor chemoattracting and activating effect on neutrophils in vitro.

Compliance with ethics guidelines

Heng LI, Xiao-fei SHEN, Xin-e ZHOU, Yan-e SHI, Lu-xia DENG, Yi MA, Xiao-ying WANG, Jing-yu LI, and Ning HUANG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Bolintineanu, D., Hazrati, E., Davis, H.T., *et al.*, 2010. Antimicrobial mechanism of pore-forming protegrin peptides: 100 pores to kill *E. coli*. *Peptides*, **31**(1):1-8. http://dx.doi.org/10.1016/j.peptides.2009.11.010

Bratton, D.L., Henson, P.M., 2011. Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol.*, **32**(8):350-357. http://dx.doi.org/10.1016/j.it.2011.04.009

Brogden, K.A., 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.*, **3**(3):238-250. http://dx.doi.org/10.1038/nrmicro1098

Cao, Y., Wu, G., Fan, B., *et al.*, 2011. High mobility group nucleosomal binding domain 2 protein protects bladder epithelial cells from *Klebsiella pneumoniae* invasion. *Biol. Pharm. bull.*, **34**(7):1065-1071. http://dx.doi.org/10.1248/bpb.34.1065

Chen, H., Wang, B., Gao, D., *et al.*, 2013. Broad-spectrum antibacterial activity of carbon nanotubes to human gut bacteria. *Small*, **9**(16):2735-2746. http://dx.doi.org/10.1002/smll.201202792

Costerton, J.W., Stewart, P.S., Greenberg, E., 1999. Bacterial biofilms: a common cause of persistent infections. *Science*, **284**(5418):1318-1322. http://dx.doi.org/10.1126/science.284.5418.1318

Degryse, B., Resnati, M., Rabbani, S.A., *et al.*, 1999. Src-dependence and pertussis-toxin sensitivity of urokinase receptor-dependent chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *Blood*, **94**(2): 649-662.

Deng, L.X., Wu, G.X., Cao, Y., *et al.*, 2011. The chromosomal protein HMGN2 mediates lipopolysaccharide-induced expression of β-defensins in A549 cells. *FEBS J.*, **278**(12): 2152-2166. http://dx.doi.org/10.1111/j.1742-4658.2011.08132.x

Deng, L.X., Wu, G.X., Cao, Y., *et al.*, 2012. The chromosomal protein HMGN2 mediates the LPS-induced expression of β-defensins in mice. *Inflammation*, **35**(2):456-473. http://dx.doi.org/10.1007/s10753-011-9335-3

Feng, Y., Huang, N., Wu, Q., *et al.*, 2005. HMGN2: a novel antimicrobial effector molecule of human mononuclear leukocytes? *J. Leukoc. Biol.*, **78**(5):1136-1141. http://dx.doi.org/10.1189/jlb.0505280

Feng, Y., He, F., Zhang, P., *et al.*, 2009. Inhibitory effect of HMGN2 protein on human hepatitis B virus expression and replication in the HepG2.2.15 cell line. *Antivir. Res.*, **81**(3):277-282. http://dx.doi.org/10.1016/j.antiviral.2008.12.011

Furusawa, T., Cherukuri, S., 2010. Developmental function of HMGN proteins. *BBA-Gene Regul. Mech.*, **1799**(1):69-73.

Hawkey, P.M., Jones, A.M., 2009. The changing epidemiology of resistance. *J. Antimicorob. Chemoth.*, **64**(Suppl. 1): i3-i10. http://dx.doi.org/10.1093/jac/dkp256

Høiby, N., Bjarnsholt, T., Givskov, M., *et al.*, 2010. Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Ag.*, **35**(4):322-332. http://dx.doi.org/10.1016/j.ijantimicag.2009.12.011

Lai, Y., Gallo, R.L., 2009. AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol.*, **30**(3):131-141. http://dx.doi.org/10.1016/j.it.2008.12.003

Lehrer, R.I., Rosenman, M., Harwig, S.S., *et al.*, 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods*, **137**(2):167-173. http://dx.doi.org/10.1016/0022-1759(91)90021-7

Liu, Y., Knapp, K.M., Yang, L., *et al.*, 2013. High in vitro antimicrobial activity of β-peptoid-peptide hybrid oligomers against planktonic and biofilm cultures of *Staphylococcus epidermidis*. *Int. J. Antimicrob. Ag.*, **41**(1):20-27. http://dx.doi.org/10.1016/j.ijantimicag.2012.09.014

Mitra, A., Palaniyandi, S., Herren, C.D., *et al.*, 2013. Pleiotropic roles of *uvrY* on biofilm formation, motility and virulence in uropathogenic *Escherichia coli* CFT073. *PLoS ONE*, **8**(2):e55492. http://dx.doi.org/10.1371/journal.pone.0055492

O'Toole, G., Kaplan, H.B., Kolter, R., 2000. Biofilm formation as microbial development. *Annu. Rev. Microbiol.*, **54**(1): 49-79. http://dx.doi.org/10.1146/annurev.micro.54.1.49

Park, B., Fikrig, S., Smithwick, E., 1968. Infection and nitroblue-tetrazolium reduction by neutrophils: a diagnostic aid. *Lancet Oncol.*, **292**(7567):532-534. http://dx.doi.org/10.1016/S0140-6736(68)92406-9

Park, C.B., Kim, H.S., Kim, S.C., 1998. Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Bioph. Res. Commun.*, **244**(1):253-257. http://dx.doi.org/10.1006/bbrc.1998.8159

Reddy, K., Yedery, R., Aranha, C., 2004. Antimicrobial peptides: premises and promises. *Int. J. Antimicrob. Ag.*, **24**(6):536-547. http://dx.doi.org/10.1016/j.ijantimicag.2004.09.005

Reeves, R., 2010. Nuclear functions of the HMG proteins. *BBA-Gene Regul. Mech.*, **1799**(1):3-14.

Rovere-Querini, P., Capobianco, A., Scaffidi, P., *et al.*, 2004. HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep.*, **5**(8):825-830. http://dx.doi.org/10.1038/sj.embor.7400205

Sarda-Mantel, L., Saleh-Mghir, A., Welling, M., *et al.*, 2007. Evaluation of 99mTc-UBI 29-41 scintigraphy for specific detection of experimental *Staphylococcus aureus* prosthetic joint infections. *Eur. J. Nucl. Med. Mol. Imaging*, **34**(8): 1302-1309. http://dx.doi.org/10.1007/s00259-007-0368-7

Steinstraesser, L., Kraneburg, U., Jacobsen, F., *et al.*, 2011. Host defense peptides and their antimicrobial-immunomodulatory duality. *Immunobiology*, **216**(3):322-333. http://dx.doi.org/10.1016/j.imbio.2010.07.003

Stewart, P.S., Costerton, J.W., 2001. Antibiotic resistance of bacteria in biofilms. *Lancet*, **358**(9276):135-138. http://dx.doi.org/10.1016/S0140-6736(01)05321-1

Tunc, O., Thompson, J., Tremellen, K., 2010. Development of the NBT assay as a marker of sperm oxidative stress. *Int. J. Androl.*, **33**(1):13-21. http://dx.doi.org/10.1111/j.1365-2605.2008.00941.x

Wang, K., Yan, J., Dang, W., *et al.*, 2014. Dual antifungal properties of cationic antimicrobial peptides polybia-MPI: membrane integrity disruption and inhibition of biofilm formation. *Peptides*, **56**:22-29. http://dx.doi.org/10.1016/j.peptides.2014.03.005

Wang, L., Rao, C., Gao, K., *et al.*, 2013. Development of a reference standard of *Escherichia coli* DNA for residual DNA determination in China. *PLoS ONE*, **8**(9):e74166. http://dx.doi.org/10.1371/journal.pone.0074166

Wiegand, I., Hilpert, K., Hancock, R.E., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.*, **3**(2):163-175. http://dx.doi.org/10.1038/nprot.2007.521

Wu, G., Cao, Y., Fan, B., *et al.*, 2011. High-mobility group

protein N2 (HMGN2) inhibited the internalization of *Klebsiella pneumoniae* into cultured bladder epithelial cells. *Acta Bioch. Bioph. Sin.*, **43**(9):680-687. http://dx.doi.org/10.1093/abbs/gmr064

Xie, Y., Fleming, E., Chen, J.L., *et al.*, 2011. Effect of proline position on the antimicrobial mechanism of buforin II. *Peptides*, **32**(4):677-682. http://dx.doi.org/10.1016/j.peptides.2011.01.010

Yang, D., Postnikov, Y.V., Li, Y., *et al.*, 2012. High-mobility group nucleosome-binding protein 1 acts as an alarmin and is critical for lipopolysaccharide-induced immune responses. *J. Exp. Med.*, **209**(1):157-171. http://dx.doi.org/10.1084/jem.20101354

Zanetti, M., 2004. Cathelicidins, multifunctional peptides of the innate immunity. *J. Leukocyte Biol.*, **75**(1):39-48. http://dx.doi.org/10.1189/jlb.0403147

Zhang, L., Wang, Y.W., Lu, Z.Q., 2015. Midgut immune responses induced by bacterial infection in the silkworm, *Bombyx mori*. *J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.)*, **16**(10):875-882. http://dx.doi.org/10.1631/jzus.B1500060

中文概要

题　目：高迁移率族蛋白 N2（HMGN2）对革兰氏阴性大肠埃希菌的抗菌机制研究

目　的：报道高迁移率族蛋白 N2（HMGN2）对大肠埃希菌（*Escherichia coli*）K12 的抗菌功能，并对其抗菌机制进行探讨，同时检验 HMGN2 对中性粒细胞是否具有趋化活性。

创新点：从分子水平上探讨了 HMGN2 对大肠埃希菌的抗菌机制。

方　法：用反相高效液相色谱法从人类子宫纤维囊腺瘤中提取组织细胞的 HMGN2 分子（tHMGN2）。诱导重组表达质粒 PET-32a-c(+)-HMGN2 表达重组蛋白 HMGN2（rHMGN2）。用琼脂糖凝胶弥散法对 HMGN2 的抗菌活性进行检测，并用微量肉汤稀释法测定 HMGN2 的最小抑菌浓度（MIC）。通过膜通透性实验和凝胶阻滞实验检测 HMGN2 对细菌菌膜和核酸的作用。通过结晶紫实验和电镜扫描验证 HMGN2 的抗生物被膜形成作用。通过氮蓝四唑（NBT）法和 Transwell 趋化法分别验证 HMGN2 的活化效应和对中性粒细胞的趋化活性。

结　果：我们分离纯化获得了高质量的天然和重组 HMGN2 分子，同时验证了 HMGN2 对革兰氏阴性大肠埃希菌具有较强的抗菌活性，MIC 为 16.25 μg/ml。细菌膜通透性实验发现 HMGN2 使大肠埃希菌膜渗透性明显增大。HMGN2 分子与大肠埃希菌 K12 染色体 DNA 和质粒 DNA 的结合均呈浓度依赖效应。银染和扫描电镜结果显示，HMGN2 与大肠埃希菌共培养可干扰细菌生物被膜形成，并破坏已形成的早期和成熟生物被膜。然而 HMGN2 对中性粒细胞没有活化作用和趋化作用。

结　论：作为抗菌肽，HMGN2 对大肠埃希菌有良好的抗菌活性。该活性可能通过影响细胞膜的通透性和干扰细菌 DNA 转录以及干扰生物被膜而发挥作用。

关键词：高迁移率族蛋白 N2（HMGN2）；膜通透性；生物被膜；趋化作用