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Nesfatin-1 inhibits ovarian epithelial carcinoma cell proliferation *in vitro*

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ABSTRACT

Nesfatin-1, an 82-amino-acid peptide derived from a 396-amino-acid precursor protein nucleobindin 2 (NUCB2), was originally identified in hypothalamic nuclei involved in the regulation of food intake. It was recently reported that nesfatin-1 is a novel depot specific adipokine preferentially produced by subcutaneous tissue, with obesity- and food deprivation-regulated expression. Although a relation between ovarian cancer mortality and obesity has been previously established, a role of nesfatin-1 in ovarian epithelial carcinoma remains unknown. The aim of the present study is to examine the effect of nesfatin-1 on ovary carcinoma cells proliferation. We found that nesfatin-1 inhibits the proliferation and growth of HO-8910 cells by G1 phase arrest, this inhibition could be abolished by nesfatin-1 neutralizing antibody. Nesfatin-1 enhances HO-8910 cell apoptosis, activation of mammalian target of rapamycin (mTOR) and RhoA/ROCK signaling pathway block the effects of nesfatin-1-induced apoptosis, therefore reverses the inhibition of HO-8910 cell proliferation by nesfatin-1. In conclusion, the present study demonstrated that nesfatin-1 can inhibit the proliferation in human ovarian epithelial carcinoma cell line HO-8910 cells through inducing apoptosis via mTOR and RhoA/ROCK signaling pathway. This study provides a novel regulatory signaling pathway of nesfatin-1-regulated ovarian epithelial carcinoma growth and may contribute to ovarian cancer prevention and therapy, especially in obese patients.

1. Introduction

Ovarian cancer is one of the most common cause of death from all cancers among women and the leading cause of death from gynecological malignancies. Ovarian epithelial carcinoma is a common malignant ovarian neoplasm with poor 5-year survival rate (less than 30%). Many factors regulate the rapid growth of ovarian epithelial carcinoma. Adipokines could be important for the development of obesity-related diseases such as diabetes, cardiovascular diseases, and cancer [1].

Nesfatin-1, an 82-amino-acid peptide derived from a 396-amino-acid precursor protein nucleobindin 2 (NUCB2), was originally identified in hypothalamic nuclei involved in the regulation of food intake such as the arcuate nucleus, lateral hypothalamus, paraventricular nucleus and supraoptic nucleus [2]. Subsequent studies revealed that nesfatin-1/NUCB2 is also distributed in peripheral tissues including the stomach, pancreatic islets, testis and adipose tissue [3,4]. It was reported that nesfatin-1 is a novel depot specific adipokine preferentially produced by subcutaneous tissue, with obesity- and food deprivation-regulated expression [5]. Secretion of nesfatin-1 into the culture media was dramatically increased during the differentiation of 3T3-L1 preadipocytes into adipocytes and after treatments with TNF-α, IL-6, insulin, and dexamethasone, circulating nesfatin-1 levels were higher in high-fat-fed mice and showed positive correlation with body mass index in human.

Although the literature to date focusing on obesity and prognosis of ovarian cancer has been inconclusive, a recent meta-analysis reported a positive relationship between early adulthood BMI and mortality among patients with ovarian cancer [6], however, the mechanism is not clear. As a novel adipokine, the role of neafatin-1 in the progress of ovarian epithelial carcinoma is still unknown.

In the present study, we reported that the proliferation could be inhibited by nesfatin-1 in human ovarian epithelial carcinoma cells and explored the mediating mechanism and possible significance.

2. Materials and methods

2.1. Chemicals and reagents

HO-8910 cell, a human ovarian epithelial carcinoma cell line, was purchased from American type culture collection (ATCC, Manassas, VA). RPMI1640 medium was purchased from Hyclone Co. (Logan, UT). Recombinant human nesfatin-1 (1–82) and anti-nesfatin neutralizing antibody were purchased from Phoenix Pharmaceuticals (Belmont, CA). Mouse-anti-β-actin antibody was

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purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were purchased from Cell Signaling Technology (Beverly, MA). All other chemicals and drugs were purchased from Sigma Chemical (St. Louis, MO).

2.2. Cell culture

HO-8910 cells, a human ovarian epithelial carcinoma cell line, were cultured in RPMI1640 containing 10% FBS and penicillin/streptomycin (100 U/ml) in a humidified 37 °C incubator. When confluent, cells were treated with nesfatin-1 (10^{-11}–10^{-8} M) for 48 h. For the inhibition experiments, cells were pretreated with L-leucine or Y27632 for 1 h prior to stimulation with nesfatin-1 at 10^{-9} M for 48 h.

2.3. RNA extraction and RT-PCR analysis

Total RNAs were isolated using Trizol reagent according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed using reverse transcription system (Promega, Madison, WI). One microliter of the reaction mixture was subjected to PCR. The forward and reverse PCR primers were: human proliferating cell nuclear antigen (PCNA) 5′-TGT TGG AGG CAC TCA AGG AC-3′, and 5′-TCA TTG CCG GCG CAT TTT AG-3′ (Genebank Sequence ID: NM_002592.2); human β-actin 5′-ATC TGG CAC CAC ACC TTC-3′ and 5′-AGC CAG GTC CAG ACG CA-3′ (Genebank Sequence ID: NM_001101.3). All amplification reactions were performed under the following conditions: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s. A 20 μl aliquot of the RT-PCR samples was loaded onto 1.5% agarose gel. For the quantitative real time PCR analysis, the amount of PCR products formed in each cycle was evaluated on the basis of SYBR Green I fluorescence. Results were analyzed with Stratagene Mx3000 software and mRNA levels were normalized with respect to the levels of β-actin in each sample.

2.4. Cell proliferation and viability assays

To determine the effect of nesfatin-1 on HO-8910 cell proliferation, 30% confluent HO-8910 cells were incubated in RPMI1640 media with different concentrations of FBS (0%, 5% and 10%) in the presence or absence of nesfatin-1 (10^{-9} M) for 48 h. On completion of the incubation, cultures were typsinized and cell numbers were determined with an Invitrogen CountessH Automated Cell Counter (Carlsbad, CA).

The WST-1 and Cell counting kit-8 (CCK-8) assays were used to determine the effect of nesfatin-1 on HO-8910 cell viability. Briefly, 1×10^3 cells/well were incubated in 96-well plates overnight, starved in serum-free medium for 24 h, and treated with indicated reagents. For WST-1 assay (BioVision Research Products, Milpitas, CA, USA), cells were incubated with 10 μL of WST-1 reagent for 45 min and absorbance was measured at 450 nm using Bio-Rad iMark Microplate Absorbance Reader (Bio-Rad, USA). For CCK-8 assay (Dojindo Molecullar Technologies Inc. Kumamoto, Japan), cells were incubated with 10 μL of CCK-8 solution for 1 h and absorbance was measured at 450 nm.

2.5. Cell cycle analysis

HO-8910 cells, cultured with or without nesfatin-1 (10^{-9} M) for 24 h, were trypsinized, fixed and permeabilized with 70% ethanol. Cells were then labeled with propidium iodide with RNase A cocktail for 30 min at 37 °C and flow cytometry was used to determine the cell cycle distribution of the HO-8910 cells. Data were obtained using FACS Caliburflow cytometer (BD Biosciences, USA).

2.6. Apoptosis analysis

HO-8910 cells were cultured in 96-well plate with or without nesfatin-1 (10^{-9} M) stimulation. Apoptosis was assessed by measuring cysteine aspartic acid-specific protease (caspase) 3/7 activity with the use of the Caspase-Glo® 3/7 Assay Kit (Promega) by following the manufacturer's instructions.

2.7. Preparation of cytosolic proteins and Western blot analysis

Following treatment, the cells were packed by centrifuging the cells for 3 min at 200 × *g*, and homogenized in ice-cold fractionation buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 mM sodium orthovanadate [Na_3VO_4], 10 mM sodium pyrophosphate [$Na_4P_2O_7$] and 50 mM sodium fluoride [NaF]). The cell lysate was incubated on ice for 15 min and then centrifuged at 20,000 × *g* for 30 min at 4 °C. The cytosolic fraction was collected and subjected to SDS–PAGE with a 10% running gel. Protein concentrations were determined by BCA Protein Assay Kit (PIERCE, Rockford, IL). The proteins were transferred to a polyvinylidene fluoride membrane. The membrane was incubated successively with 5% bovine serum albumin in tris tween buffered saline (TTBS) at room temperature for 1 h, with different first antibodies at 4 °C for 12 h and then with horseradish peroxidase-labeled second antibody for 1 h. After each incubation, the membrane was washed extensively with TTBS, and the immunoreactive band was detected with ECL-detecting reagents (PIERCE, Rockford, IL).

2.8. RhoA activation assay

HO-8910 cells were cultured in 96-well plate with or without nesfatin-1 (10^{-9} M) stimulation. RhoA activity was measured using a G-LISA® RhoA Activation Assay Biochem Kit™ (Cytoskeleton, Denver, CO) according to manufacturer's instructions.

2.9. Statistical analysis

Quantitative data are presented as the means ± SEM determined from the indicated number of experiments. Statistical analysis was based on Student's *t*-test for comparison of two groups or one-way ANOVA for multiple comparisons. $P < 0.05$ was used to determine statistical significance.

3. Results

3.1. Nesfatin-1 inhibits HO-8910 cell proliferation

We first study the possible role of nesfatin-1 in ovarian epithelial carcinoma progression. HO-8910 cells, the human ovarian epithelial carcinoma cell line, were cultured without (Control) or with nesfatin-1 (10^{-9} M) for 48 h with different concentrations of fetal bovine serum (FBS). Treatment of HO-8910 cells with nesfatin-1 serum-freely resulted in a statistically significant decrease in cell number compared to control (Fig. 1A). Treatment with increasing amounts of serum resulted in a concentration-dependent increase in cell number. The inhibitory effect of nesfatin-1 was still significant with increasing concentrations of serum in the cell culture medium. Flow cytometry was used to evaluate the effect of nesfatin-1 on the HO-8910 cell cycle progression. Nesfatin-1 (10^{-9} M) significantly increased the portion of cells in the G1 phase by approximately 37.2% ($P < 0.01$) (Supplementary data), and the portion of G2/M and S phase cells was decreased by a comparable

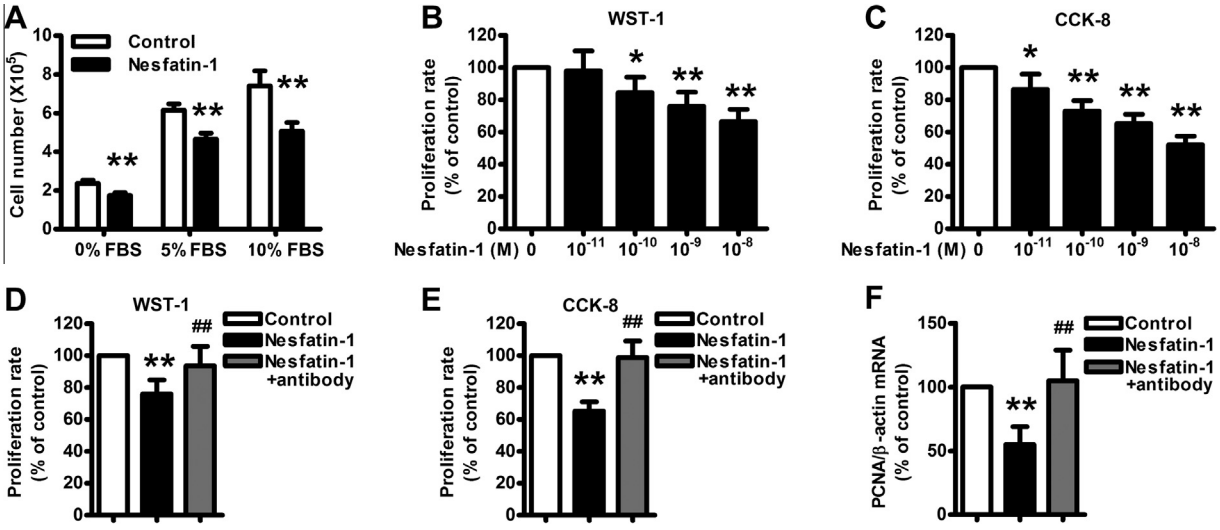

Fig. 1. Effect of nesfatin-1 on HO-8910 cell proliferation. (A) HO-8910 cells were treated without (control) or with nesfatin-1 (10^{-9} M) supplied with increasing amounts of serum for 48 h and cell numbers were counted. (B, C) HO-8910 cells were treated with indicating dosages of nesfatin-1 for 48 h and cell viability was detected with WST-1 and CCK-8 assay. (D, E) Augmentation of nesfatin-1-inhibited proliferation by anti-nesfatin-1 neutralizing antibody. Cells were pretreated with or without neutralizing antibody (1:1000) for 1 h, then incubated in the presence of nesfatin-1 (10^{-9} M) for 48 h. (F) Expression of PCNA in HO-8910 cell treated by nesfatin-1 and anti- nesfatin-1 neutralizing antibody. Relative mRNA levels were normalized to that of untreated cells. Data are means ± SEM from 4 separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus nesfatin-1-untreated cells, $^{\#\#}P < 0.01$ versus nesfatin-1 treatment alone.

degree, which indicated that nesfatin-1 could inhibit effectively the proliferation and growth of HO-8910 cells by G1 phase arrest.

The WST-1 and CCK-8 assays also showed that nesfatin-1 treatment (10^{-11}–10^{-8} M) for 48 h caused a concentration-dependent inhibition in HO-8910 cell proliferation (Fig. 1B and C), with maximal inhibition to around 66.6% and 52.1% of control found with 10^{-8} M of nesfatin-1. The expression of PCNA, the novel proliferation-related gene usually highly expressed in G1/S phase, was also inhibited by nesfatin-1 significantly (Fig. 1F). Neutralizing antibody of nesfatin-1 significantly attenuated the HO-8910 cell proliferation (Fig. 1D–F), which indicates that nesfatin-1-inhibited HO-8910 cell proliferation depends on its immune activity.

3.2. Nesfatin-1 promotes apoptosis in HO-8910 cells

The members of the caspase family play key effector roles in apoptosis in mammalian cells. We then detected the effect of nesfatin-1 on the apoptosis of HO-8910 cells. As expected, nesfatin-1 (10^{-9} M) increased the production of apoptosis marker, cleaved caspase-3, significantly (Fig. 2A). The activity of caspase-3/7 was also increased significantly by nesfatin-1 treatment (Fig. 2B). These effects were depended on nesfatin-1 immune activity since neutralizing antibody of nesfatin-1 significantly attenuated the effect of nesfatin-1 on HO-8910 cell apoptosis (Fig. 2A and B).

3.3. mTOR signaling pathway is involved in nesfatin-1-inhibited proliferation

The mammalian target of rapamycin (mTOR) is a central cell-growth regulator that regulates cell proliferation and aberrant mTOR activity is linked to the development of cancer [7]. In the present study, we found that there was a significant decrease in phosphorylated mTOR (Ser2448) under nesfatin-1 treatment (10^{-9} M), so did the phosphorylation of S6 ribosomal protein, a downstream target of mTOR (Fig. 3A). Administration of L-leucine (1, 5, 10 mM), a branched-chain amino acid that has been documented to activate mTOR signaling [8], significantly restored nesfatin-1-inhibited HO-8910 cell proliferation (Fig. 3B–D). Administration of L-leucine (5 mM) also significantly attenuated nesfatin-1-induced HO-8910 cell apoptosis (Fig. 3E). Taken together, nesfatin-1 inhibited HO-8910 cell proliferation through inhibition of mTOR signaling pathway.

3.4. RhoA/ROCK pathway participated in nesfatin-1-stimulated apoptosis and proliferation

RhoA is a small GTPase that interacts with a downstream effector, Rho-associated coiled coil-containing protein kinase (ROCK), to control many cellular processes, including apoptosis

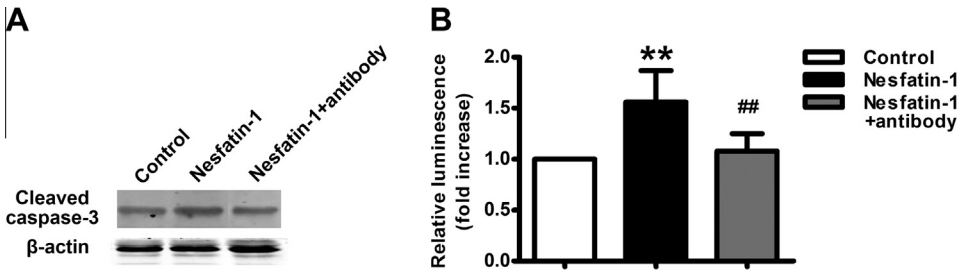

Fig. 2. Nesfatin-1 promotes apoptosis in HO-8910 cells. (A) Western blot analysis of nesfatin-1-induced cleaved caspase-3 protein levels in HO-8910 cells. (B) Caspase-3/7 activity detected in HO-8910 cells treated with nesfatin-1. Cells were pretreated with anti-nesfatin-1 neutralizing antibody for 1 h, and then with nesfatin-1 (10^{-9} M) for 48 h. Data are means ± SEM from 4 separate experiments. $^{**}P < 0.01$ versus nesfatin-1-untreated cells, $^{\#\#}P < 0.01$ versus nesfatin-1 treatment alone.

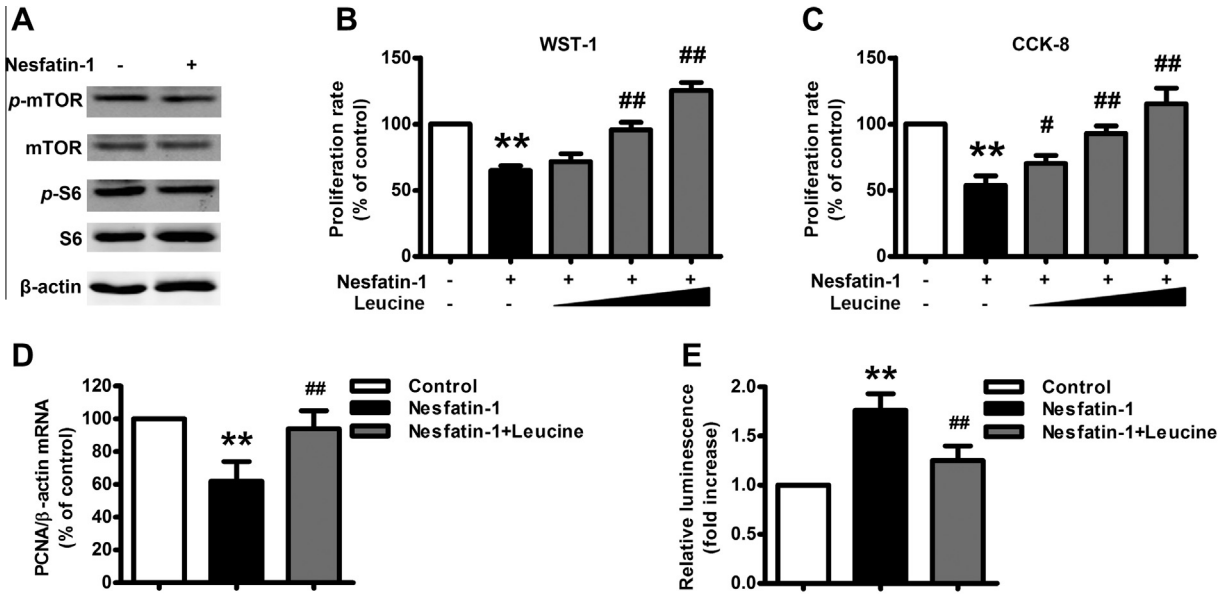

Fig. 3. mTOR signaling pathway is involved in nesfatin-1-inhibited proliferation. (A) Effects of nesfatin-1 on mTOR pathway activation in HO-8910 cells. Cells were incubated with nesfatin-1 (10^{-9} M) for 6 h and whole-cell lysates underwent western blotting to detect the phosphorylation of mTOR (Ser2448) and S6. (B, C) Augmentation of nesfatin-1-inhibited proliferation by L-leucine (1, 5, 10 mM) under WST-1 and CCK-8 assays. (D) Attenuation of nesfatin-1-inhibited PCNA expression by L-leucine in HO-8910 cells. (E) Blockage of nesfatin-1-induced caspase-3/7 activity by L-leucine in HO-8910 cells. Cells were pretreated with or without L-leucine (5 mM) for 1 h, and then incubated in the presence of nesfatin-1 (10^{-9} M) for 48 h. Data are means ± SEM from 4 separate experiments. $^{**}P < 0.01$ versus nesfatin-1-untreated cells, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus nesfatin-1 treatment alone.

[9,10]. To determine whether RhoA/ROCK pathway is involved in nesfatin-1-induced apoptosis, we first examined the effect of nesfatin-1 on a RhoA activity in HO-8910 cells. Nesfatin-1 (10^{-9} M) significantly increased relative RhoA activity level in HO-8910 cells (Fig. 4A). To address the potential role of RhoA/ROCK pathway in nesfatin-1-induced apoptosis in HO-8910 cells, Y27632, a pharmacological inhibitor of RhoA/ROCK, was used. Pretreatment of Y27632 (10 μM) for 1 h significantly attenuated nesfatin-1-induced HO-8910 cell apoptosis (Fig. 4E) and augmented nesfatin-1-inhibited HO-8910 cell proliferation (Fig. 4B–D). Taken together, RhoA/ROCK pathway mediated nesfatin-1-induced apoptosis, which further regulated proliferation.

4. Discussion

The present study demonstrated that nesfatin-1 can inhibit the proliferation in human ovarian epithelial carcinoma cell line HO-8910 cells through inducing apoptosis via mTOR and RhoA/ROCK signaling pathway and subsequently contribute to cancer prevention and therapy. This conclusion is supported by the following observations: (1) Nesfatin-1 inhibits the proliferation and growth of HO-8910 cells by G1 phase arrest; (2) Nesfatin-1 enhances HO-8910 cell apoptosis; (3) Activation of mTOR signaling pathway blocks the effects of nesfatin-1 on apoptosis, therefore reverses the inhibition of HO-8910 cell proliferation; (4) Activation of RhoA/

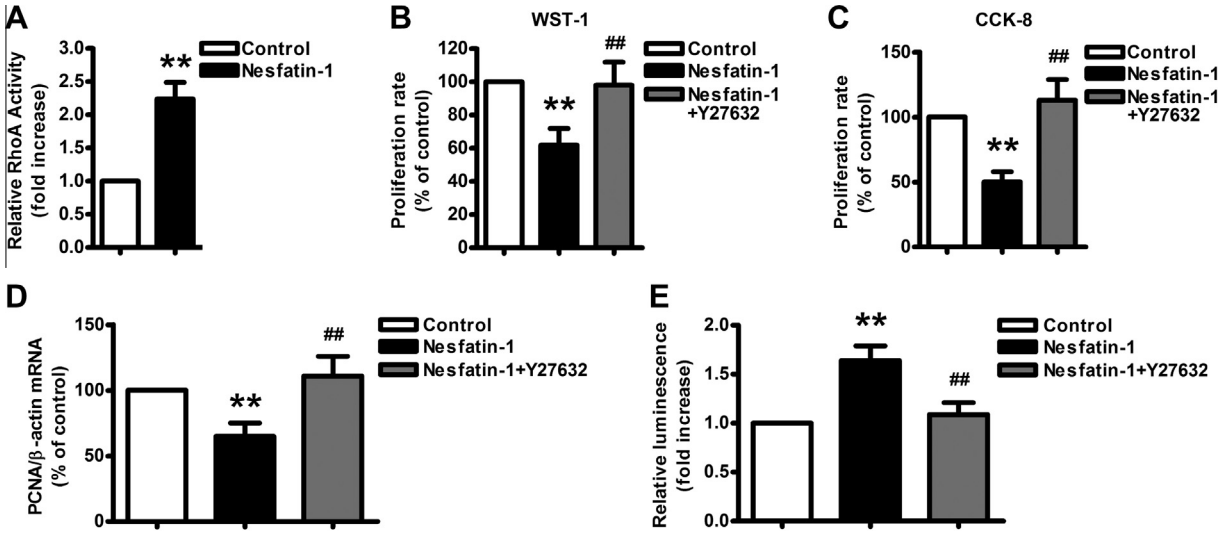

Fig. 4. RhoA/ROCK pathway participated in nesfatin-1-stimulated apoptosis in HO-8910 cells. (A) Relative RhoA activity in HO-8910 cells treated with or without nesfatin-1 (10^{-9} M) for 12 h. (B–D) Augmentation of nesfatin-1-inhibited proliferation by RhoA/ROCK inhibitor. (E) Attenuation of nesfatin-1-induced caspase-3/7 activity detected in HO-8910 cells treated with RhoA/ROCK inhibitor. Cells were pretreated with RhoA/ROCK inhibitor Y27632 (10 μM) for 1 h, and then with nesfatin-1 (10^{-9} M) for 48 h. Data are means ± SEM from 4 separate experiments. $^{**}P < 0.01$ versus nesfatin-1-untreated cells, $^{\#\#}P < 0.01$ versus nesfatin-1 treatment alone.

ROCK signaling pathway blocks the effects of nesfatin-1 on apoptosis, therefore reverses the inhibition of HO-8910 cell proliferation. To the best of our knowledge, this is the first report demonstrating the involvement of multiple signaling pathways in the nesfatin-1-mediated modulation of proliferation in ovarian epithelial carcinoma.

Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States, although it is the 12th most common cause of cancer death among women in China, the mortality is more than 70% within 5 years. A relation between ovarian cancer mortality and obesity has been previously established, obesity impacts ovarian cancer mortality by influencing tumor biology, significant differences were found in the risk of ovarian cancer progression and ovarian cancer-related mortality associated with increasing BMI in a fairly "dose-dependent" fashion [11]. Adipose tissue is now broadly recognized as a genuine endocrine organ secreting several bioactive adipocytokines, which regulate physiological and pathological processes, such as insulin sensitivity and resistance, inflammation, immunity, and angiogenesis [12,13]. As a newly confirmed adipokine, the effect of nesfatin-1 on the pathogenesis and progress of malignant tumor, especially in ovarian carcinoma, is rarely reported. In the present study, we confirmed the effect of nesfatin-1 on the proliferation of ovarian epithelial carcinoma cells, although the receptor is not defined yet, nesfatin-1 inhibits HO-8910 cell proliferation dependent on its immune activity.

The mammalian target of rapamycin (mTOR), a ubiquitously expressed protein kinase and important regulator of cell growth and proliferation, is implicated in cell processes that lead to uncontrolled growth of cancer cells. L-Leucine is a specific activator of mTOR [8], downstream targets of mTOR include S6 kinases, S6, and eIF-4E binding protein 1 [14,15]. Exactly how mTOR contributes to cancer is still unclear. It is thought that mTOR and its downstream signals affects cell proliferation and tumorigenesis by promoting the translation of specific mRNAs coding for pro-oncogenic proteins regulating cell survival, cell-cycle progression, angiogenesis, energy metabolism, and metastasis [16]. Additionally, the increase in ribosome biogenesis linked to mTOR activation probably promotes cell proliferation by providing the machinery required to sustain high levels of cell growth. A number of agents that target the mTOR pathway have shown potent antitumorigenic effects [17]. The effect of nesfatin-1 on mTOR activity is controversial, nesfatin-1 was reported to elicit a marked upregulation or downregulation of the hypothalamic mTOR signaling pathway. In the present study, we reported the downregulation of activities of mTOR and its downstream signal S6 under nesfatin-1 treatment in HO-8910 cells. This downregulation mediated nesfatin-1-inhibited HO-8910 cell proliferation. Two mTOR complexes are known to exist: mTOR complex 1 (mTORC1) is responsible for nutrient-sensing functions and is composed of mTOR, G protein-subunit-like protein, and raptor; mTORC2 phosphorylates Akt protein kinase B and contains mTOR and rictor [15]. In the present study, we found that L-leucine significantly attenuated nesfatin-1-induced HO-8910 cell apoptosis and therefore increase proliferation, indicating that mTORC1 mediated the effect of nesfatin-1. The role of mTORC2 during the nesfatin-1 effect is still unclear.

Programmed cell death plays a fundamental role in animal development and tissue homeostasis [18]. Abnormal regulation of this process is associated with a wide variety of human diseases, including immunological and developmental disorders, and cancer. Apoptosis is the most important form of programmed cell death. In the present study, we reported that nesfatin-1 induced the apoptosis of HO-8910 cells and inhibited tumor growth subsequently. However, although nesfatin-1 inhibited proliferation of HO-8910 cells, it is also reported that apoptosis might also induce a compensative proliferation under certain environment [19]. The balance of nesfatin-1-regulated proliferation and apoptosis needs further discussion.

The small Rho GTPase family of proteins, encompassing the three major G-protein classes RhoA, Rac and cell division control protein 42, are key mitogenic signaling molecules that regulate multiple cancer-associated cellular phenotypes including cell proliferation and motility. RhoA/ROCK pathway played an important role in cancer cell proliferation and invasion [20]. These proteins are known for their role in the regulation of actin cytoskeletal dynamics, which is achieved through modulating the activity of their downstream effector molecules. It has been discovered that the ROCK kinases actively phosphorylate a large cohort of actin-binding proteins and intermediate filament proteins to modulate their functions. As a consequence, they regulate many key cellular functions associated with malignancy, including cell proliferation, motility and viability. Here, we report that nesfatin-1 treatment evoked a marked activation of RhoA; nesfatin-1-induced RhoA-ROCK activity enhanced apoptosis and inhibited proliferation, which indicated that RhoA-ROCK pathway is benefit for controlling tumor growth under nesfatin-1 stimulation.

In summary, the present study demonstrated that nesfatin-1 can inhibit the proliferation in human ovarian epithelial carcinoma cell line HO-8910 cells through inducing apoptosis via mTOR and RhoA/ROCK signaling pathway. This study provides a novel regulatory signaling pathway of nesfatin-1-regulated ovarian epithelial carcinoma growth and may contribute to ovarian cancer prevention and therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.001.

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