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Original Article

siRNA directed against Annexin II receptor inhibits HeLa cell proliferation, migration and invasion and induces apoptosis via suppressing ERK1/2 and Akt signaling pathways

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Abstract: Annexin II receptor (AXIIR) was originally identified as a cell surface receptor for Annexin II and was shown to be involved in the development and progression of multiple cancers, but little was known about its role in cervical cancer. The aim of our study was to investigate the role and the plausible molecular mechanism of AXIIR in regulating apoptosis, invasion and migration of cervical cancer cells. siRNA targeting AXIIR was chemical synthesized and transfected into HeLa cells which with non-specific siRNA tranfection used as a negative control. Cell proliferation, cell migration, cell invasion and cell apoptosis assay were measured by CCK-8, wound healing assay, Transwell invasion assay and flow cytometry, respectively. Tumor-related protein and signaling pathway were also measured by real-time quantitative PCR and western blot assay to investigate the molecular mechanism of AXIIR involved in regulating HeLa cells. Our data showed that AXIIR siRNA significantly inhibited HeLa cell viability and induced cell apoptosis by increases in Caspase-3/-8/-9 mRNA and protein expression, and inhibit HeLa cell migration and invasion by decreased MMP-2/-9 mRNA and protein expression. AXIIR siRNA could also significantly down-regulate Akt, p-Akt, ERK1/2 and p-ERK1/2 expression. In conclusion, AXIIR is of great importance for regulatingcell viability, migration, invasion and apoptosis *in vitro*. The molecular mechanism of AXIIR might be the activation of PI3K/Akt and ERK1/2 signaling pathway, indicating a critical regulator function of AXIIR in tumorigenic responses of cervical cancer.

Keywords: Cervical cancer, HeLa, AXIIR, siRNA, PI3K/Akt, ERK1/2

Introduction

Cervical cancer is one of the most common cancers in women with difference in age and background throughout the world and is the fourth-most common cause of death from cancer. Women who are diagnosed with cervical cancerwas estimated to more than 529,800 cases each year, and approximately 275,100 women die from cervical cancer each year [1]. Although early-stage cervical cancer can be treated by radical surgery with or without chemotherapy and/or radiotherapy, invasive cervical cancer is still a major health issue in the whole world and some patients with high risk factors have an unfavorable prognosis [2]. The 5-year survival rates for stage III and IV patients remain at less than 40% [3]. Moreover, approximately 20% to 30% of patients experience lymph node recurrence and distant metastasis after primary treatment [4]. Despite the causes of cervical cancer have not yet been fully elucidated, human papillomavirus (HPV) infection is a major risk factor for cervical cancer and that more than 80% of patients are positive for high-risk HPV (HPV16, HPV18 and HPV31) [5, 6]. The molecular mechanisms in regard of the carcinogenesis ofcervical cancer are still not well understood. Therefore, it is necessary to increase the understanding of the molecular targets and pathways in progression and metastasis of cervical cancer.

Annexin II (AXII) is a member of a family of proteins that bind to anionic phospholipid surfaces in the presence of calcium [7], and has been

implicated in multiple intracellular and extracellular processes [8, 9]. Annexin II expression is increased in multiple cancers, including glioma [10], prostate [11], gastric [12], colorectal [13], and lung cancer [14] as well as cervical cancer [15] and predicted a poor survival outcome. AXII may act as a receptor of HPV16 involved in HPV16-induced infection in cells [16, 17]. Inhibition of AXII could complete blockage of HPV16 infection in HeLa cells, suggesting a novel target for treatment of cervical cancer [18], whereas AXII receptor (AXIIR) may play an important role in it. AXIIR is only detected in primate species [19] and regulates growth and motility of prostate cancer cells and multiple myeloma cells [20, 21]. Previous studies have been reported that AXIIR induced chronic myeloid leukemia cell apoptosis in an AXII-independent manner [22, 23], suggesting that AXIIR may not be the specific receptor of AXII [24]. Despite little known about the reliable functions of AXIIR currently, it is of great important significance that AXIIR implicates in a broad range of cellular functions. Consequently, developing an effective application strategy required a detailed understanding of the function of AXIIR for treatment of cervical cancer.

In our study, the effect of AXIIR on cell viability, motility and apoptosis of HeLa were investigated. We found that AXIIR knockdown significantly inhibited HeLa cell viability, migration and invasion and induced cell apoptosis. In order to better understand the molecular mechanism, the expression levels of MMP-2, MMP-9, Caspase-3, Caspase-8, and Caspase-9 as well as activation of ERK1/2 and Akt in HeLa cells with AXIIR knockdown were also measured. We found AXIIR-siRNA could inhibit activation of ERK1/2 and Akt, decrease expression of MMP-2, MMP-9 and increase Caspase-3, Caspase-8 and Caspase-9 expression. Our results demonstrate an important role of AXIIR in regulation of HeLa cell apoptosis, invasion and migration.

Materials and methods

Reagents

Dulbecco's modified eagle medium (DMEM)-high glucose (4.5 g/L), fetal bovine serum (FBS), non-essential amino acid, glutamine, streptomycin, penicillin G and 0.25% trypsin were purchased from GibcoLife Technologies (Grand Island, NY, USA). Negtive control-siRNA and AXIIR-siRNA were purchased from GenePharma (Shanghai, China). Lipofectamine 2000, Opti-MEM, protein marker, PVDF membrane and HRP-IgG were purchased from Invitrogen (Paisley, UK). Antibodies against AXIIR, β-actin and GAPDH were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against Akt, p-Akt, ERK1/2, and p-ERK1/2 were purchased from Bioworld Technology, Inc. (Louis Park, MN, USA). Cell counting kit (CCK)-8 and Annexin V-FITC/PI Apoptosis Detection Kit were obtained from Beyotime Institute of Biotechnology, Inc. (Shanghai, China).

Cell line and cell culture conditions

The HeLa cell line was procured from the Cell Bank of Academia Sinica (Shanghai, China), and maintained in DMEM-high glucose (4.5 g/L) supplemented with 10% FBS, 1% non-essential amino acid, 1% glutamine and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37°C in a humidified incubator (BD Biosciences, San Diego, CA, USA) containing 5% CO_2 and 95% air.

siRNA chemical synthesis and transfection

AXIIR-siRNA (sense strand, 5'-CCACCUAUUGUGAGUUCAGTT-3'; antisense strand, 5'-CUGAACUCACAAUAGGUGGTT-3'). Negative control non-specific siRNA (sense strand, 5'-UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3'). HeLa cells were grown in six-well plates and transfected with a mixture of 1 μl AXIIR siRNA/negative control siRNA, 50 μl Opti-MEM and 2 μl Lipofectamine 2000, according to the manufacturer's instructions. After 6-8 h, cells were washed and cultured for 24 h in a complete medium.

Cell viability assay

The effects of AXIIR-siRNA on HeLa cell viability were evaluated by MTT assay. Briefly, HeLa cells (2×10^5 cells/well) were seeded and transfected with or without AXIIR-siRNA. After 24 htransfection, the medium was changed and cells were incubated in 10% FBS-DMEM for 48 h, followed by MTT 5 mg/ml added for 2 h at 37°C, and then dimethyl sulfoxide (DMSO) was added into each well for 30 min. The cell viability was calculatedusing a microplate reader

Table 1. Primes sequences used in this study

Gene	Sequences
MMP-2-forward	5'-TTGACGGTAAGGACGGACTC-3'
MMP-2-reverse	5'-GGCGTTCCCATACTTCACAC-3'
MMP-9-forward	5'-AAGGGCGTCGTGGTTCCAACTC-3'
MMP-9-reverse	5'-AGCATTGCCGTCCTGGGTGTAG-3'
Caspase-3-forward	5'-AACTGGACTGTGGCATTGAG-3'
Caspase-3-reverse	5'-ACAAAGCGACTGGATGAACC-3'
Caspase-8-forward	5'-TGGAGTTAGGCAGGTTAG-3'
Caspase-8-reverse	5'-GGGTTCTTGCTTCCTTTG-3'
Caspase-9-forward	5'-CCTCACCCTGCCTTATCTTG-3'
Caspase-9-reverse	5'-TCCCTCTTCCTCCACTGTTC-3'
GAPDH-forward	5'-CACCCACTCCTCCACCTTTG-3'
GAPDH-reverse	5'-CCACCACCCTGTTGCTGTAG-3'

(ELX 800; Bio-tek Instruments, Winooski, VT, USA) at a wavelength of 490 nm.

Cell apoptosis assay

The apoptotic rate of HeLa cells induced by AXIIR-siRNA tranfection was measured by flow cytometry assay. In brief, HeLa cells transfected with or without AXIIR-siRNA were seeded at a density of 2×10^5 cells/ml and washed with 1 ml Binding Buffer (3 ml 10× Binding Buffer in 27 ml ddH_2O). Subsequently, the cells were stained with 5 μl Annexin V-FITC for 10 min in dark room and with 5μl Propidium Iodide (PI) for 5 min. Apoptotic cells were analyzed by flow cytometry, by use of a FACScan system flow cytometric analysis (Becton-Dickinson FACS Calibur, San Joes, CA, USA) equipped with Cell Quest 3.3 software.

Wound healing assay for cell migration

HeLa cells transfected with or without AXIIR-siRNA were seeded in 12-well plate and cultured overnight until 30%-40% confluent, and the monolayer with a 100-μl pipette tip was scratched and then incubated in fresh medium for 12, 24, 36 and 48 h. Cell debris were removed by washing twice with the serum-free medium. Then, the cells which migrated into the wounded area were visualized and photographed under a phase contrast microscope (Olympus, Tokyo, Japan). Three different areas in each assay were selected to measure the distance of the migrating cells to the origin of the wound.

Transwell invasion assay

The ability of HeLa cells to pass through Matrigel-coated filters was measured using a Transwellinvasion assay. Briefly, Matrigel-coated inserts of 8-μm-pore size (BD Biosciences, Madrid, Spain) were placed into 24-well plates at 37°C in a 5% CO_2 incubator for 1 h. The HeLa cells transfected with or without AXIIR-siRNA were serm-starved for 24 h, following which 5×10^4 cells in 500 μl serum-free DMEM were placed in the upper chamber at 37°C. The medium containing 10% FBS (750 μl) was added into the lower chamber. After 48 h incubation, cells remaining on the upper surface of the membrane were removed with a cotton swab. The cells crossing the Matrigel to the lower surface of the membrane were washed, fixed and stained. The invasive cells found on the bottom site of each inserts were then photographed and counted under a microscope (×200; CX41RF; Olympus Corporation, Tokyo, Japan).

RNA extraction and real-time reverse transcription polymerase chain reaction

Total RNA from the HeLa cells was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and 2 μg of RNA was reverse transcribed with PrimeScript RT reagents Kit according to the manufacturer's instructions. Real-time PCR was carried out using SYBR Green (Takara, Otsu, Shiga, Japan) and performed using the GeneAmp PCR Systems 2700 (Applied Biosystems). The primer sequences of the genes used in quantitative PCR were listed in **Table 1**. Expression values were calculated using the comparative Ct method and the GAPDH gene was used as endogenous control.

Protein extraction and Western blot analysis

Total protein from the HeLa cells was extracted with RIPA lysis buffer supplemented with protease inhibitors. Twenty micrograms of total protein was separated through 5% or 12.5% SDS-PAGE, and the gel was electrophoretic transferred onto polyvinylidene difluoride membrane. Blots were incubated with anti-MMP-2, anti-MMP-9, anti-Caspase-3, anti-Caspase-8, anti-Caspase-9, anti-AXIIR, anti-Akt, anti-p-Akt, anti-ERK1/2, and anti-p-ERK1/2 antibody overnight at 4°C, followed by 3 washes of 5 to 7

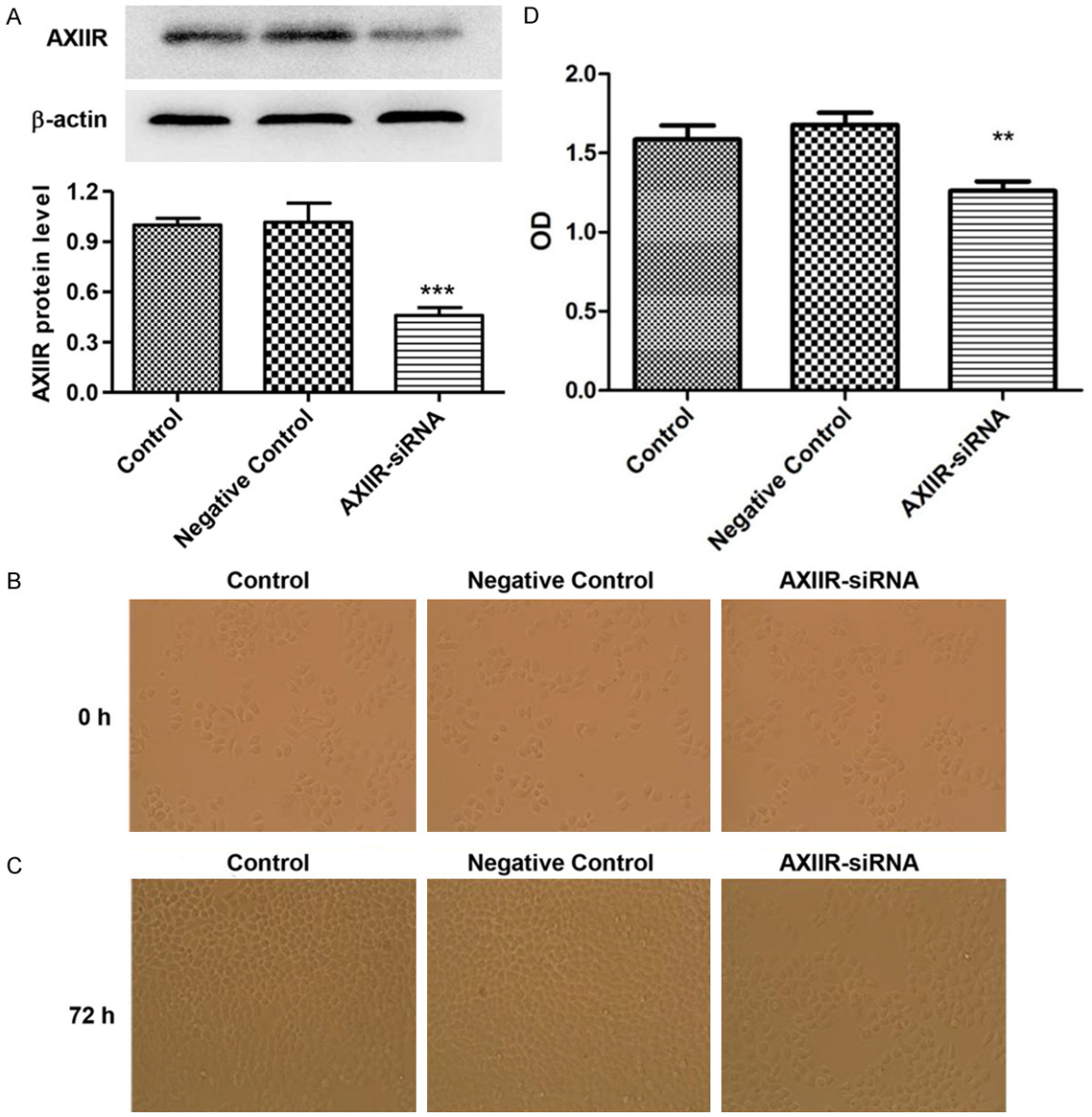

Figure 1. AXIIR-siRNA inhibited cell proliferation of HeLa cells. (A) Western blot analysis of AXIIR expression in HeLa cells with AXIIR-siRNA or negative control siRNA transfection. HeLa cell morphology was measured after transfection with AXIIR-siRNA or negative control siRNA for 0 h (B) and 72 h (C). (D) The cell viability was measured by MTT assay. ***P<0.001 compared with control.

minutes each in TBST. Blots were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature, washed 3 times for 5 to 7 min in TBST. The blots were visualized by using Ultrasensitive ECL Chemiluminescence Kit (Sangon Biotech, Shanghai, China) and exposed to X-ray film and quantified in Chemi Doc XRS Imaging System, Bio-Rad (USA).

Statistical analysis

Data is expressed as mean ± SD of triplicate experiments through analyzing with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and statistical analysis was performed with the Student's t test. Significance of difference was designated as $P<0.05$.

Results

AXIIR-siRNA inhibits cell viability in HeLa cells

Because malignant tumors were characterized by the abnormal survival and growth of tumor cells, the effects of AXIIR knockdown on the viability of HeLa cells with transfection of siRNA

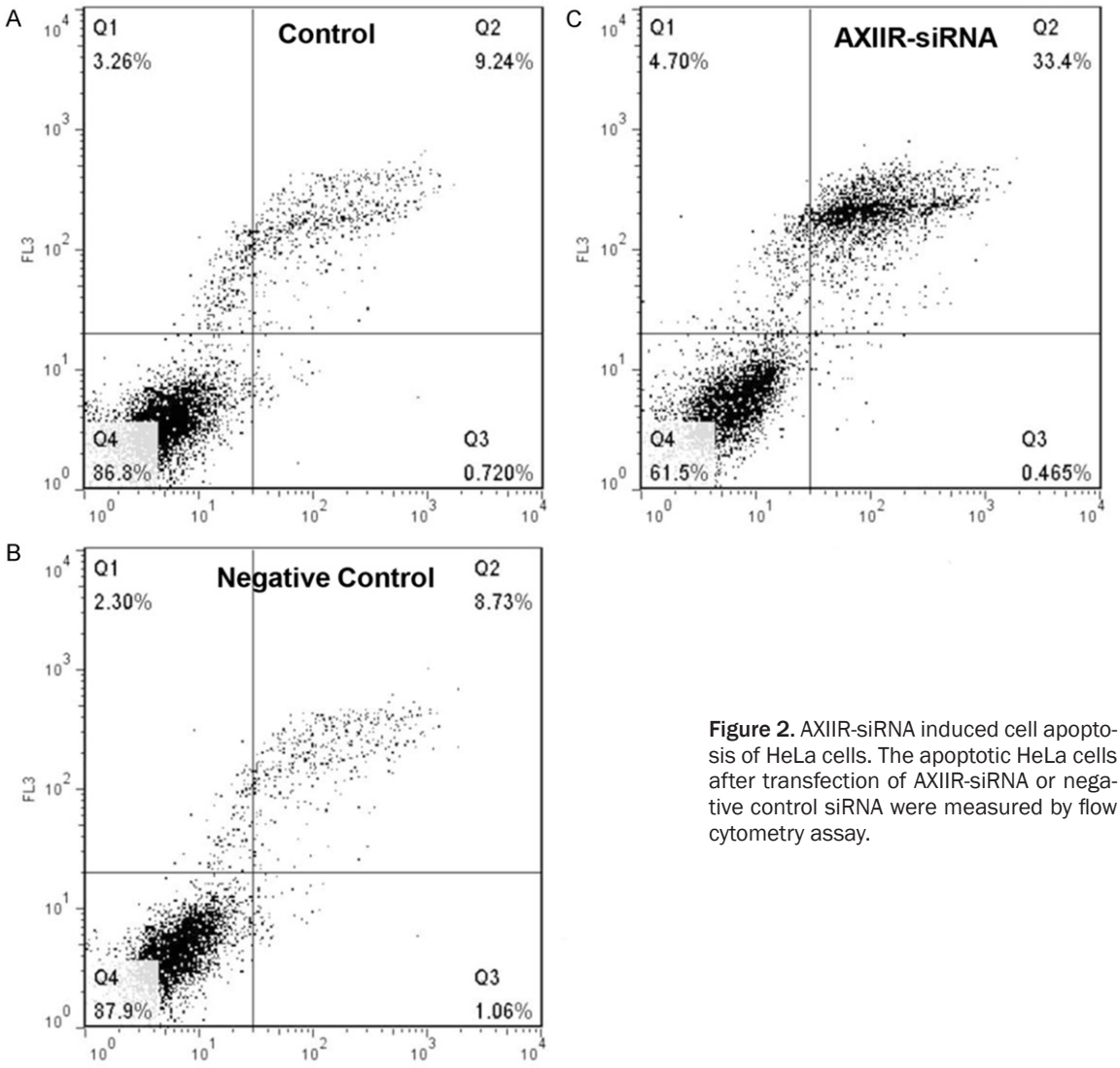

Figure 2. AXIIR-siRNA induced cell apoptosis of HeLa cells. The apoptotic HeLa cells after transfection of AXIIR-siRNA or negative control siRNA were measured by flow cytometry assay.

directly targeted AXIIR were firstly investigated. After 48 h of transfection, Western blotting was performed to examine the protein levels of AXIIR in HeLa cells, cell morphology was observed under an inverted light microscope, and MTT assay was performed to detect cell viability. As shown in **Figure 1A**, AXIIR knockdown significantly decreased the protein expression levels of AXIIR compared with the control group, while there were no differences between negative control and control groups. AXIIR knockdown also inhibited cell growth ability and affected cell morphology of HeLa cells compared with control groups (**Figure 1B** and **1C**). Moreover, HeLa cells with AXIIR-siRNA transfection showed markedly decreased cell viability compared to control groups (**Figure 1D**). Our data suggest that AXIIR knockdown could remarkably suppress cell viability of human cervical cancer cells.

AXIIR-siRNA induces apoptosis in HeLa cells

To further investigate the possible mechanism of AXIIR-siRNA inhibition of cell viability, the function of AXIIR-siRNA on cell apoptosis was also measured by using Annexin V-FITC/PI staining and flow cytometry analysis. The results showed an increased cell apoptosis of HeLa after treatment with AXIIR-siRNA compared with two other groups (**Figure 2A** and **2B**). In addition, HeLa cells with AXIIR-siRNA transfection observed 33.9% of apoptotic cells, whereas only 9.9% and 9.8% in negative control and blank groups, respectively. Our data suggest that AXIIR knockdown could remark-

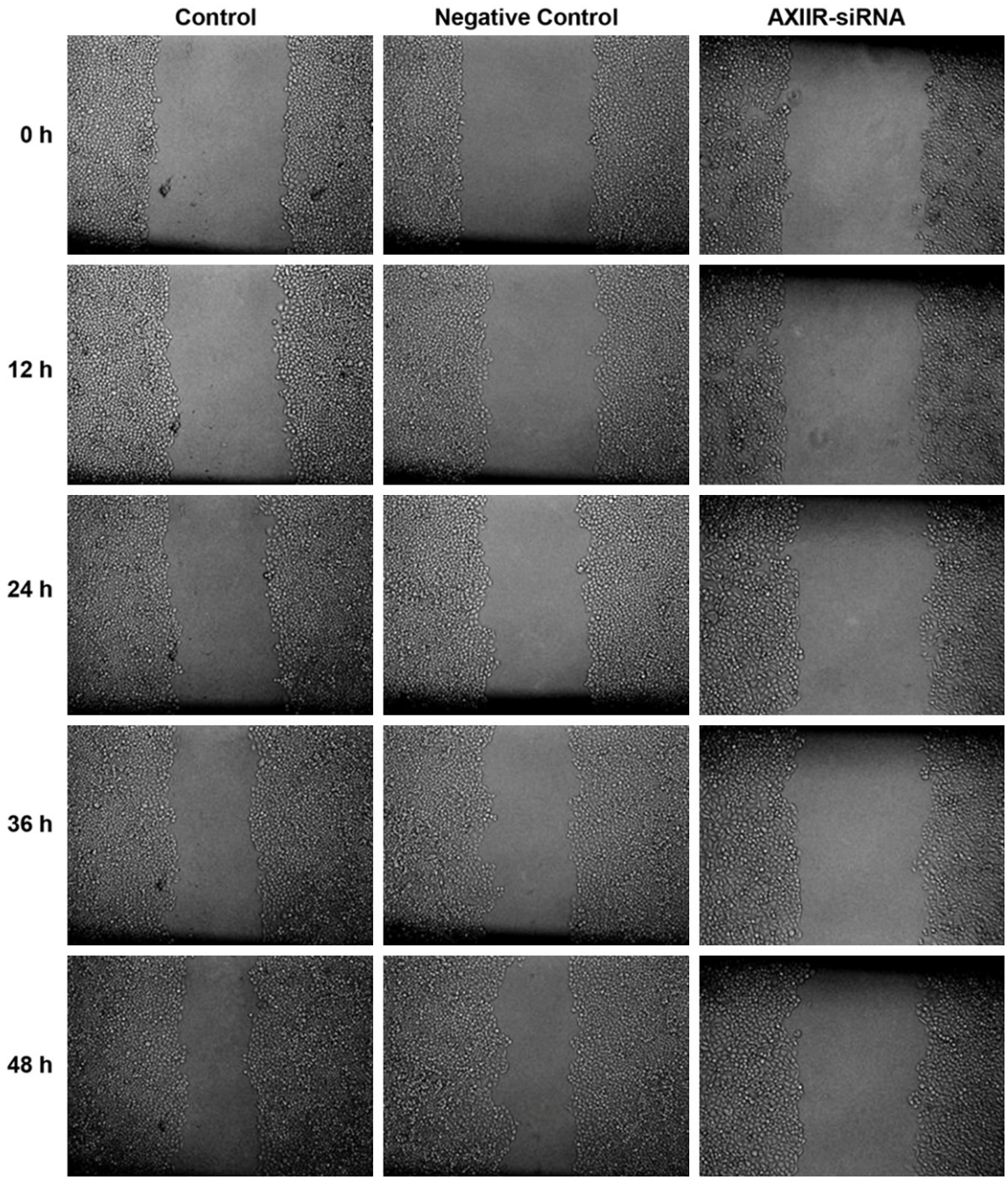

Figure 3. AXIIR-siRNA inhibited cell migration of HeLa cells. Cell migration was measured by Wound healing assay after 0, 12, 24, 36, 48 h transfection and representative images of the migrated cells were shown.

ably induce cell apoptosis of human cervical cancer cells.

AXIIR-siRNA inhibits cell migration in HeLa cells

Cell migration plays an important role intumor cell metastasis. We next investigated the effect of AXIIR knockdown on HeLa cell migration. We found that a wound scratch in untreated and negative control cells was practically entirely closed after 12, 24, 36 and 48 h of incubation (**Figure 3**). Nonetheless, HeLa cells with AXIIR-siRNA transfection observed marked decrease in wound healing in compared with two other groups. Our data suggest that AXIIR knockdown

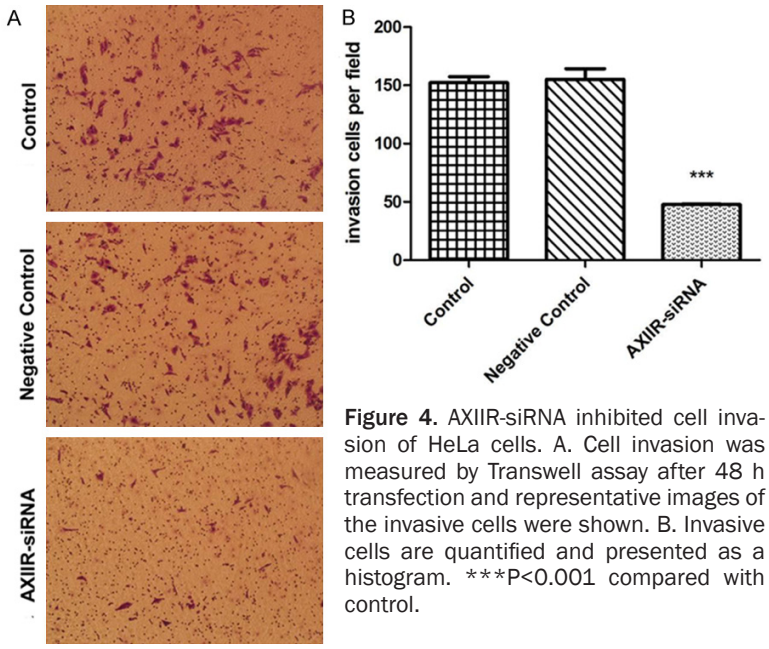

Figure 4. AXIIR-siRNA inhibited cell invasion of HeLa cells. A. Cell invasion was measured by Transwell assay after 48 h transfection and representative images of the invasive cells were shown. B. Invasive cells are quantified and presented as a histogram. ***P<0.001 compared with control.

could inhibit cell migration in human cervical cancer cells.

AXIIR-siRNA inhibits cell invasion in HeLa cells

To define the biological effect of AXIIR down-regulation on metastasis in human cervical cancer cells, transwell assay was also performed. Consistent with wound healing assay, the Matrigel-coated transwell assay confirmed that AXIIR-siRNA markedly inhibited the invasive capacity of the HeLa cells compared with two other groups (**Figure 4A** and **4B**). AXIIR-siRNA transfection remarkably decreased the number of invasive cells by 66.7% compared with control groups. Our data suggest that AXIIR knockdown could inhibit cell invasion in human cervical cancer cells.

AXIIR-siRNA inhibits MMP-2/9 and increases caspase-3/8/9 expression in HeLa cells

To investigate the molecular pathway of AXIIR regulating the tumorigenesis of human cervical cancer, the expression of apoptosis, migration and invasion-related genes, MMP-2, MMP-9, Caspase-3, Caspase-8 and Caspase-9 was measured by Real-time PCR and Western blot assay. We found that AXIIR knockdown significantly reduced the expression of MMP-2 and MMP-9 (**Figure 5A**, **5B**, **5F**) and increased the expression of Caspase-3, Caspase-8 and Caspase-9 both at mRNA level and protein level (**Figure 5C-F**) in HeLa cells compared with two other groups. Our data suggest that AXIIR knockdown could induce apoptosis by increasing Caspase-3, Caspase-8 and Caspase-9, and inhibit invasion and migration by decreasing MMP-2 and MMP9 in human cervical cancer cells.

AXIIR-siRNA inhibits ERK1/2 and Akt activation in HeLa cells

Previous studies have been shown that ERK1/2 and Akt signaling pathways were implicated in regulating the activities of MMP-2/9 [25] as well as Caspase-3/8/9 [26, 27]. To evaluate whether the decreased MMP-2/9 levels and the increased expression of Caspase-3/8/9 was mediated by the inhibition of ERK1/2 and Akt pathways, the protein expression of ERK1/2, p-ERK1/2, Akt and p-Akt was measured by Western blotting. As shown in **Figure 6**, AXIIR-siRNA significantly decreased the protein expression of ERK1/2, p-ERK1/2, Akt and p-Akt compared with two other groups. In addition, AXIIR-siRNA also inhibited the activation of ERK1/2 and Akt, evidenced by decreased ratio of p-ERK1/2/ERK1/2 and p-Akt/Akt. Our data suggest that AXIIR knockdown could inhibit activation of ERK1/2 and Akt in human cervical cancer cells.

Discussion

Being the fourth most common malignant tumor among women in the developing countries, human cervical cancer has attracted researchers' attention generally; however, the therapeutic outcome of cervical canceris incapable of satisfactory, so that new strategies need to be exploited, in which molecular therapy may be of great important. In the present study, we found for the first time that knockdown of AXIIR could significantly inhibit HeLa cell viability, migration and invasion, and induce apoptosis through ERK1/2 and Akt-mediated down-regulated MMP-2/9 and up-regulated Caspase-3/8/9-dependent pathway.

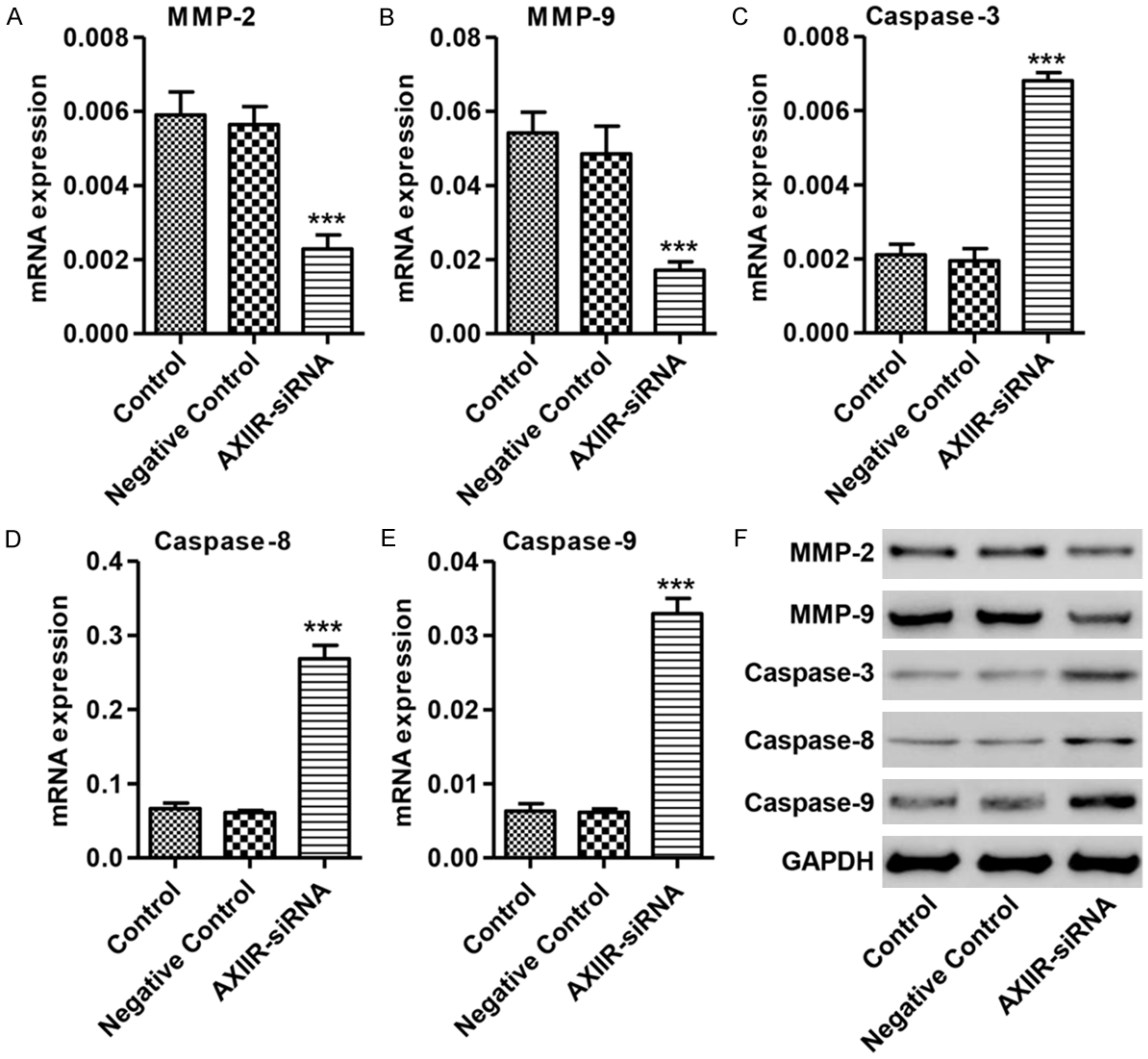

Figure 5. AXIIR-siRNA inhibited MMP-2/9 expression and increased Caspase-3/8/9 expression of HeLa cells. The expression of MMP-2, MMP-9, Caspase-3, Caspase-8 and Caspase-9 in HeLa cells was measured after transfection with AXIIR-siRNA or negative control siRNA by RT-PCR (A-E) and Western blotting (F), respectively. ***P<0.001 compared with control.

Annexins are calcium-dependent and membrane-binding high-abundant intracellular proteins which show cell type-specific expression [28, 29]. Annexin II is a member of the Annexins family implicated in multiple intracellular and extracellular processes, including cell proliferation, membrane physiology, tumor invasion, metastasis and angiogenesis [20, 21]. Previous studies have demonstrated that AXIIR as a receptor for Annexin II involved in mediating Annexin II signal. It is noteworthy that AXIIR couldinhibitcell apoptosis in multiple human cancers, including multiple myeloma [21] and prostate cancer [20]. In consistent with the previous studies, AXIIR knockdown by siRNA-marked decrease in cell viability and increase of cell apoptosis of HeLa cells, suggesting a pro-proliferative role of AXIIR in HeLa cells. However, AXIIR overexpression inhibited cell viability and induced apoptosis in uveal melanoma [30]. The data suggest that the function of AXIIR might vary in different cell types. The intrinsic and extrinsic pathways are two major apoptotic pathways. The cytochrome C and AIF release and the activation of caspase-9 contribute to the intrinsic pathway [31, 32]; while caspase-8 activation is responsible for the extrinsic pathway [33]. In the present study, we found that Caspases-3, Caspases-8 and Caspases-9 expression was significantly increa-

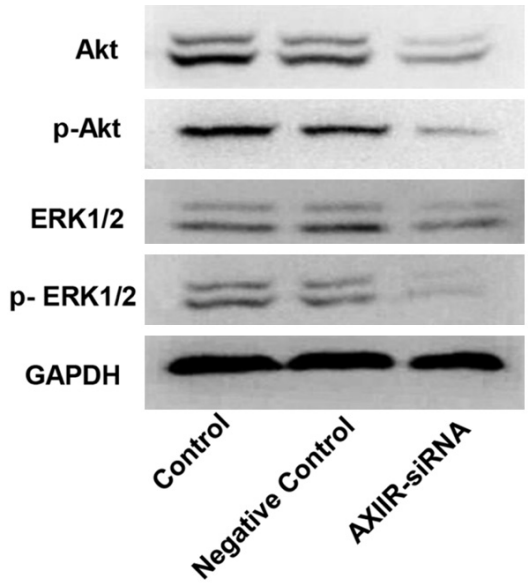

Figure 6. AXIIR-siRNA inhibited ERK1/2 and Akt activation in HeLa cells. The expression of p-ERK1/2, p-Akt, ERK1/2 and Aktin HeLa cells after transfection with AXIIR-siRNA or negative control siRNA was measured by Western blotting, respectively.

sed in response to AXIIR-siRNA induced apoptosis, suggesting the involvement of both intrinsic and extrinsic apoptotic pathways in AXIIR-mediated apoptosis.

Furthermore, this is the first report on the function of AXIIR ininducing migration and invasion in HeLa cells. In line with the previous study that AXIIR knockdown by using RNA interference could suppress the migration and adhesion of HUVECs *in vitro* and angiogenesis *in vivo,* indicating an pro-angiogenesis effect of AXIIR in HUVECs [23]. AXII/AXIIR plays a critical role in the progression of prostate cancer metastasis and therapeutic intervention targeting prostate cancer metastasis may give the possibility to improve the prognosis of prostate cancer patients [20]. To further investigate the molecular mechanism of AXIIR regulating migration and invasion, the pro-migrated and pro-invasive factors regulated by AXIIR siRNA were examined. Our results showed that AXIIR knockdown could down-regulation of the mRNA and protein levels of MMP-2 and MMP-9 expression. MMP-2 and MMP-9 are the major matrix metalloproteinases (MMPs) mediated by a variety of signal transduction pathways regulating and controlling tumor metastasis [34]. These findings indicated a critical function of AXIIR in degradation of the extracellular matrix.

Functional assays demonstrated that AXIIR siRNA stimulated the apoptosis and inhibited invasion and migration of HeLa cells was through the inactivation of ERK1/2 and Akt. Both ERK1/2 and Akt activation have been previously shown to regulate cell proliferation, apoptosis, angiogenic potential, invasion and migration of cervical cancer [26, 35]. In the present study, AXIIR siRNA markedly inhibited activation of ERK1/2 and Akt in HeLa cells. Inhibition of ERK1/2 significantly induced HeLa cell apoptosis through the activation of Caspase-3/-8 pathway, which is agreement with our findings [26]. Moreover, AXIIR knockdown inhibited angiogenesis through decreasing phosphorylation levels of ERK1/2 and Akt thus reducing MMP-2 and MMP-9 expression [23].

In conclusion, we demonstrated that AXIIR was essential in regulating cell viability, apoptosis, migration and invasion *in vitro*. Moreover, we found that the molecular mechanism might be the increase in Caspase-3/8/9 and inhibition of expression of MMP-2/9 which resulted from the inhibition of ERK1/2 and Akt pathways and thereby suggest novel targets for therapeutic intervention to prevent cervical cancer.

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Disclosure of conflict of interest

None.

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