SiRNA Directed Against Annexin II Receptor Inhibits Angiogenesis via Suppressing MMP2 and MMP9 Expression

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Abstract
Background/Aims: Annexin II receptor (AXIIR) is able to mediate Annexin II signal and induce apoptosis, but its role in angiogenesis remains unclear. This study tries to investigate the role of AXIIR in angiogenesis and the plausible molecular mechanism. Methods/Results: RNA interference technology was used to silence AXIIR, and the subsequent effects in vitro and in vivo were evaluated thereafter. Our data indicated that human umbilical vein endothelial cells (HUVECs) expressed AXIIR and knockdown of AXIIR significantly inhibited HUVECs proliferation, adhesion, migration, and tube formation in vitro and suppressed angiogenesis in vivo. Furthermore, AXIIR siRNA induced cell arrest in the S/G2 phase while had no effect on cell apoptosis. We found that these subsequent effects might be via suppressing the expression of matrix metalloproteinase 2 and matrix metalloproteinase 9. Conclusion: AXIIR participates in angiogenesis, and may be a potential therapeutic target for angiogenesis related diseases.

Introduction

Angiogenesis is a process that new blood capillaries sprouting from pre-existing ones which is of great physiological and pathological importance [1]. It is a complicated process composed of multiple steps that include Endothelial cells (ECs) activation, guided sprouting proliferation, branching coordination, lumen formation, perfusion and vessel maturation [2].

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The excessive or insufficient angiogenic switch may lead to many kinds of diseases. Malignant, ocular and inflammatory disorders are the best-known pathological processes result from undue angiogenesis while ischemic heart disease, preedampsia or vessel malformation are the most common disorders result from insufficient angiogenesis [3]. Therefore, it is of great significance to understand the molecular mechanisms of angiogenesis thoroughly which will shed new light on angiogenesis related disease therapy.

AXIIR, also known as Chromosome 5 open reading frame 39 (C5orf39), is originally cloned from human marrow cDNA, and identified as a receptor for Annexin II. AXIIR gene encodes a protein with 193 amino acids which is only detected in primate species up to now [4]. As a member of the Annexins family, Annexin II involves in many cell functions such as endocytic and exocytotic vesicular transport, interaction with cell adhesion molecules, regulation of ion channels, and being receptor for tenascin-C and plasminogen [5-7]. Since AXIIR is originally identified as a receptor for Annexin II, several studies have investigated the role of AXIIR in mediating Annexin II signal. The researchers find that AXIIR can interact with Annexin II to regulate cancer cell adhesion, migration and growth [8, 9]. However, some investigations suggest that AXIIR may not be the specific mediator of Annexin II signal [10]. It is noteworthy that AXIIR is found to be able to induce apoptosis in multiple human cell types and this property is Annexin II independent [11]. These findings indicate that AXIIR may involve in cell function in a more broad way while not only mediate Annexin II signal.

In the present study, we investigated the role of AXIIR in regulation of angiogenic properties of HUVECs. We found AXIIR was expressed in HUVECs and knockdown of AXIIR expression using RNA interference technique could inhibit HUVECs proliferation, adhesion, migration and tube formation significantly. Furthermore, knockdown of AXIIR was able to suppress angiogenesis in nude mice. In order to better understand the molecular mechanism, we studied the affection of AXIIR siRNA on cell cycle, cell apoptosis and Matrix metalloproteinase 2 (MMP2), Matrix metalloproteinase 9 (MMP9) expression in HUVECs. We found AXIIR siRNA could induce cell cycle arrest in the S/G2 phase while had no effect on cell apoptosis and could inhibit MMP2 and MMP9 expression significantly. The results obtained from this study demonstrate an important role of AXIIR in regulation of angiogenesis.

**Materials and Methods**

**Materials**

Fetal bovine serum, Trizol, Lipofectamine 2000 and HRP-conjugated mouse and rabbit secondary antibodies were obtained from Invitrogen (Shanghai, China). Endothelial Cell Medium (ECM) was obtained from ScienCell (San Diego, USA). The anti-AXIIR and anti-CD31 antibody were obtained from Santa Cruz Biotechnology Co., Ltd. (Dallas, USA). Antibodies to MMP2, MMP9 were obtained from Shanghai RuianBioTechnologies (Shanghai, China). The anti-AKT, anti-p-AKT, anti-ERK, anti-p-ERK, anti-TIMP2 antibodies were purchased from Bioworld Technology, Inc. (Nanjing, China). The anti-GAPDH antibody was obtained from Tianjin sungene Biotech (Tianjin, China). Matrigel Matrix was obtained from BD Biosciences (San Jose, USA). Cell-light EdU DNA cell proliferation kit was obtained from RiboBio (Guangzhou, China). PrimeScript RT reagents Kit, PrimeSTAR® HS DNA Polymerase with GC Buffer and SYBR Premix Ex Taq were obtained from TAKARA BIOTECHNOLOGY (Dalian, China). Annexin V-FITC Apoptosis Detection Kit was obtained from eBioscience (San Diego, CA, USA). The transwell chamber and other cell culture plates were obtained from Corning Inc (NY, USA). All other regents were of analytical grade.

**Cell culture**

HUVECs were obtained from ScienCell (San Diego, USA) and cultured in Endothelial Cell Medium (ECM) supplemented with 10% FBS, 1% L-glutamine and were kept in a 5% CO2 incubator at 100% humidity and with the temperature of 37°C. Passages 3 to 8 were used in all experiments.

**Reverse transcription-PCR (RT-PCR)**

Detection of AXIIR mRNA in HUVECs was performed using RT-PCR. Total RNA from HUVECs was extracted using Trizol reagent and then was reverse transcribed with PrimeScript
RT reagents Kit according to the manufacturer’s protocol. Primer sequences for AXIR were as follows (5’→3’): TCTAGAGCGCCACCCATGGAGCAACATTTCCTTG (forward) and GTGCACTAGGCTGATGCTACACAGATCG (reverse). PCR reaction was performed using PrimeSTAR® HS DNA Polymerase with GC Buffer which gives the PCR product of 582 bp. Finally, the products were analyzed by 2% agarose gel electrophoresis.

RNA interference studies

The siRNA was chemical synthesized by Shanghai GenePharma Co., Ltd. The company provided several sequences of negative control and we chose one that the composition of bases were highly similar to experimental group. The target sequence against AXIR selected were sense CCACCUAUUGUGAGUUCAGTTG and anti-sense CUGAACUCACAAUAGGUGGTG. The sequence of Negative control was sense UUCUCCGAACGUGACACGUdTdT and anti-sense ACGUGACACGUUCGAAGAdTdT. siRNA transfection was performed using Lipofectamine 2000 Reagent according to the manufacturer’s instructions and the concentration used in the subsequent experiments was 50 nmol/L.

Cell proliferation assay

Cell proliferation was determined by 5-ethynyl-2'-deoxyuridine (EdU) assay as previous described [12]. In brief, 5×10^5 cells were seeded in 96-well plates and incubated overnight. Then, the cells were treated with AXIR siRNA or controls for 48 h. We then dumped the medium and added 100 μl fresh medium containing EdU (100 μM). After incubation for 2 hours, the cells were stained as the following protocol: discard the EdU medium and fix the cells with 4% paraformaldehyde for 30 minutes. Then, wash with glycine for 5 minutes, followed by twice washes with 0.2% Trion X-100 of 10 minutes each time. Then, the cells were stained with Apollo fluorescent azide for 30 minutes, followed by three time washes of 0.2% Trion X-100. Furthermore, the cells were stained with Hoechst for 15 minutes at room temperature and washed with PBS for two times, and add 100 μl PBS to further analysis. The images were taken and analyzed using a digital microscope system (IX81, Olympus).

Cell migration assay

For the cell migration assay, 1×10^5 HUVECs treated with AXIR siRNA for 48 h were seeded in the upper chamber of transwell units with 8 μm pore size polycarbonate filter in 0.5% FBS medium. The lower chamber was filled with 700 μl ECM containing 1% FBS. After incubation for 12 hours, the grown medium was removed and the filters were fixed with 4% paraformaldehyde for 20 minutes. Then, cells on the upper surface of the filter were completely removed by wiping with a cotton swab. Furthermore, the filters were stained with 0.1% Crystal violet for 15 minutes. The number of cells that migrated through the upper to the lower surface of the filter were counted and analyzed with a digital microscope system (IX81, Olympus).

Cell adhesion assay

After treatment with AXIR siRNA for 48 h, 5×10^4 HUVECs were harvested and seeded in a 96-well plate for 8 h, unattached cells were rinsed gently with phosphate buffered saline (PBS). The remaining attached cells were fixed with 4% paraformaldehyde for 20 minutes. Then, the cells were stained with 0.1% Crystal violet for 15 minutes. Finally, the attached cells were counted and analyzed with a digital microscope system.

Tube formation assay

The tube formation assay was assessed following the protocols provided by the manufacturer. Briefly, 50 μl Matrigel was added to 96-well plate in each well and incubated at 37°C for 30 minutes for hardening. HUVECS treated with AXIR siRNA for 48 h were harvested by trypsin and resuspended in 4×10^4 cells per ml with ECM. 100 μl culture medium containing cells were gently added to the Matrigel-coated plates and incubated at 37°C for 4 hours. The capillary-like structures were then photographed with a digital microscope system.

In vivo Matrigel plug angiogenesis assay

HUVECs treated with AXIR siRNA for 12 h were harvested, and then a total of 1×10^7 HUVECs in 50 μl ECM mixed with 250 μl Matrigel were subcutaneously injected into one site for four nude mice. Matrigel mixed with the cells polymerized to form a solid gel plug later. After inoculation for 7 days, the gel plugs
was removed, fixed with formalin, embed in paraffin and section onto slides. The slides were stained with hematoxylin and eosin and anti-CD31 for immunohistochemistry. The CD31 positive staining was then photographed with a digital microscope system.

**Cell cycle assay**

Cultured HUVECs treated with AXIIR siRNA for 48h were harvested using trypsin, washed in PBS, and fixed in 70% ethanol overnight. The fixed cells were then centrifuged to remove ethanol and washed with PBS for twice. HUVECs were resuspended in 0.5 ml PBS containing 20 μg/ml propidium iodide and 200μg/ml RNase and then incubated for 30 minutes at room temperature. The samples were analyzed using a MACSQuant Analyzer (MiltenyiBiotec, Teterow, Germany). The data were then analyzed using the Modfit software.

**Cell apoptosis assay**

The cell apoptosis assay was performed using Annexin V-FITC Apoptosis Detection Kit following the manufacturer’ protocol. Cells treated with AXIIR siRNA for 48h were harvested to perform the assay. The samples were analyzed using MACSQuant Analyzer and the data were analyzed by FLOWJO v7.6 software.

**Western blot analysis**

Total protein of HUVECs was extracted in a modified Buffer with 0.5% SDS in the presence of proteinase inhibitor cocktail. Forty micrograms of proteins were electrophoresed in12.5% SDS/PAGE mini gels and transferred onto polyvinylidenefluoride membranes. The nonspecific sites were blocked with 5% nonfat milk before the membranes were incubated with primary antibodies at 4°C overnight. Then, the blots were washed 3 times for 5 to 7 minutes each time in TBS-T. Blots were incubated with horseradish peroxidase-conjugated antibodies for 2 hours at room temperature, followed by 3 washes of 5 to 7 minutes each time in TBS-T. Finally, blots were incubated with Super Signal West Pico chemoluminescent substrate and visualized using the GeneGnome HR Image Capture System (Syngene, Frederick, MD, USA).

**Real-time quantitative PCR analysis**

Total RNA from HUVECs was extracted using Trizol reagent. Then, total RNA was reverse transcribed with PrimeScript RT reagents Kit. Equal quantities of cDNA were subjected to real-time PCR with using SYBR Premix Ex Taq while GAPDH was used as the control. Reactions were incubated at 95°C for 2 minutes, followed by 40 cycles at 95°C for10 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Reactions were run on Rotor Gene 3000A (Corbett Research, Australia) and the results were expressed as the mean relative value compared with control samples. The primers used are (5´→3´): GACACAGAAGCAGCAAGA (forward) and GCGAAGAATCCACTCCAG (reverse) for AXIIR, CAGCCAACTACGATGATGA (forward) and GTGCCAAGGTCAATGTCA (reverse) for MMP2 and AACCAATCTCACCGACAG (forward) and GGCAAGTCTTCCGAGTAG (reverse) for GAPDH.

**Statistical analysis**

The data were analyzed using the GraphPad Prism 5 software, and the data were presented as the Means Standard Deviation (SD). One-way ANOVA and Tukey test were used for evaluate the data. A significance level of p<0.05 was supposed to be statistically significant.

**Results**

**Expression of AXIIR in HUVECs and its role in cell proliferation**

There is no report on the expression of AXIIR in HUVECs and its role in angiogenesis. In this study, RT-PCR and Western blotting was used to detect the mRNA and protein expression of AXIIR in HUVECs (Fig. 1A, B). The AXIIRsiRNA could cause obvious reduction of AXIIR protein expression, where control groups showed no change (Fig. 1C). Cell proliferation assay showed that AXIIRsiRNA could significantly inhibit the proliferation of HUVECs (Fig. 1D-E), which indicated that AXIIR was essential in cell proliferation.
AXIIR siRNA inhibits cell migration and adhesion

Cell migration is one of the key processes for endothelial cells to form new blood vessels in angiogenesis. We investigated whether knockdown of AXIIR would influence HUVECs migration ability. Treatment with AXIIR siRNA caused a significant decrease in the number of migrated cells compared to control groups (Fig. 2A, 2B). Since attachment of ECs to extracellular matrix is an important step that affects cell migration, we then examined the affection of treatment with AXIIR siRNA on cell adhesion capability. We found that knockdown of AXIIR could significantly reduce the number of attached cells (Fig. 2C, Fig. 2D). Altogether, the data suggested that AXIIR was required for ECs migration and adhesion.

AXIIR siRNA inhibits angiogenesis in vitro and in vivo

Another key step that involves in the process responsible for angiogenesis is tube formation of ECs. To examine the potential effects of AXIIR on endothelial cells tube formation, we studied how AXIIR affects HUVECs tube formation using two-dimensioned Matrigel assay. Compared with the control group, HUVECs treated with AXIIR siRNA showed a significant decrease in tube formation ability (Fig. 3A, B). In the in vivo Matrigel plug assay, knockdown of AXIIR was able to reduce anti-CD31 antibody-stained microvessel signals in nude mouse gel plugs (Fig. 3C, D). The result was consistent with tube formation assay in vitro, and suggested that AXIIR played a significant role in angiogenesis.

AXIIR siRNA induces cell cycle arrest while has no effect on cell apoptosis

Cell cycle is a complicated process happened in a cell which determines its division and duplication. The cell cycle plays an important role in cell growth as the cell must experience
Fig. 2. **AXII R** siRNA inhibits HUVECs migration and adhesion. (A) Representative phase-contrast images of the migrated cells. (B) Migrated cells are quantified and presented as a histogram (n=3). (C) Representative phase-contrast images of the attached cells. (D) Attached cells are quantified and presented as a histogram (n=3). The data (mean ± SD) are statistically significant where ***p < 0.001 of three separated experiments.

Fig. 3. **AXII R** siRNA inhibits angiogenesis *in vitro* and *vivo*. (A) Representative phase-contrast images of the tubes that formed. (B) The tube formation ability is quantified and presented as a histogram (n=3). (C) Representative phase-contrast images of the *in vivo* Matrigel plug CD31 IHC staining (indicated by black arrows). (D) Summary of the relative blood vessel formation ability (n=4). The data (mean ± SD) are statistically significant of three separated experiments, ***p < 0.001, **p < 0.01.
four different phases named G1 phase, S phase, G2 phase and M phase to proliferate [13]. Apoptosis is a process of programmed cell death by which the body could eliminate abnormal cells to maintain a better status [14]. To determine whether AXIIR siRNA could change the cell cycle distribution and induce cell apoptosis, we performed flow cytometry at 48h after siRNA interference. The results showed an increased cell population in the S-phase while no change in cell apoptosis after treatment with AXIIR siRNA (Fig. 4A, Fig. 4B, Fig. 4C, and Fig. 4D). The data indicated that AXIIR siRNA exhibited an inhibitory effect on cell growth through induction of S-phase cycle arrest but not through induction of cell apoptosis of HUVECs.

AXIIR siRNA suppresses pro-angiogenic factors expression to inhibit angiogenesis

In addition, the molecular pathway in which AXIIR was involved in regulating angiogenesis was determined by investigating the transcription level and protein level of angiogenesis-related genes, MMP2 and MMP9. Our data showed that compared with control groups, HUVECs treated with AXIIRsiRNA exhibited a significant decrease in the expression of MMP2 and MMP9 both at mRNA level and protein level (Fig. 5A, Fig. 5B). Furthermore, AXIIRsiRNA was able to increase the expression of tissue inhibitor of metalloproteinase 2 (TIMP2), which was regularly accompanied with the suppression of MMP2 and MMP9 (Fig. 5C) [15]. Many studies had indicated that ERK1/2 and AKT were involved in activities of MMP2 and MMP9 [16-18]. To assess whether the suppressed expression of MMP2 and MMP9 was mediated by the inhibition of AKT and ERK1/2 pathways, we investigated the effect of AXIIRsiRNA on phosphorylation of AKT and ERK1/2. We found that AXIIRsiRNA was able to suppress the activation of AKT and ERK1/2 significantly (Fig. 5C).
Discussion

Being critical to many disorders’ occurrence and development, angiogenesis has drawn investigators’ attention broadly. It has been reported that cell ingredients are able to regulate angiogenesis efficiently. The AXIIIR, a newly identified protein, may be a promising one. We found for the first time that HUVECs express AXIIIR and knockdown of AXIIIR could significantly inhibit angiogenesis which might be through down-regulating the expression of MMP2 and MMP9.

AXIIIR is expressed in most human cell types and mediates Annexin II signal by the activation of ERK1/2 and AKT pathways and induce apoptosis through the activation of Caspase-8 [4, 8, 9, 11]. However, the expression of AXIIIR in HUVECs and its role in angiogenesis have not been investigated up to now. Different from the previous report that the protein level of AXIIIR could hardly detected by Western blotting and the mRNA was easily to detect, we found that both the protein and mRNA of AXIIIR could be detected in HUVECs (Fig. 1A, B) [11]. The data suggest that HUVECs express AXIIIR abundantly, and that the expression level and function of AXIIIR might vary in different cell types.

Angiogenesis is a complicated process that composed of multiple steps of which ECs differentiation, proliferation, migration, adhesion and tubulogenesis are of great importance [1, 19]. The cell proliferation assay, cell migration assay, cell adhesion assay and tube formation assay are mature methods to imitate the angiogenesis process in vitro. The in vivo Matrigel plug assay is a mature method to imitate the angiogenesis process in vivo [20-22]. Our data showed that AXIIIRsiRNA was able to inhibit HUVECs proliferation, migration, adhesion, tube formation in vitro and inhibit angiogenesis in vivo (Fig. 1D, Fig. 2A, Fig. 2B, Fig. 3A, Fig. 3B). These results suggested a novel aspect for the role of AXIIIR in HUVECs as a positive regulator of angiogenesis. The role of AXIIIR in angiogenesis is consistent with that of Annexin II as we reported previously [23], which suggested that the function of AXIIIR in angiogenesis might be Annexin II dependent. Yet, when over-expressing AXIIIR in HUVECs using plasmid vector, we found the vector could induce apoptosis as well (unpublished data), which was Annexin II independent as reported [11]. Therefore, the relationship of Annexin II and AXIIIR in angiogenesis remains indistinct which needs further investigation.
To further reveal the inhibitory properties of AXIIRsiRNA, cell apoptosis and cell cycle assay were performed using flow cytometry. Apoptosis is a process of programmed cell death by which the body was able to eliminating abnormal cells thus maintaining a well state [14, 24]. Although over-expression of AXIIR could induce cell apoptosis, our results demonstrated that knockdown of AXIIR had no effect on cell apoptosis (Fig. 4A, Fig. 4B). The cell cycle is a series of events leading to cell division and duplication. It plays a crucial role in regulating cell proliferation thus involving the angiogenesis process [24, 25]. The data showed a remarkably increase of the percentage of cells of S phase (Fig. 4C, Fig. 4D). The result indicated that AXIIR siRNA could induce cell arrest in the S/G2 phase where synthesis of DNA and centrosomes take place.

In order to better understand the molecular mechanism of AXIIR siRNA mediated suppression of angiogenesis, we investigated the pro-angiogenic factors AXIIR siRNA regulated. MMPs are a family of zinc-dependent endopeptidases which play important roles in various physiological and pathological processes [26, 27]. Of all the MMPs, MMP2 and MMP9 are the major ones involved in angiogenesis [28-31]. We found that AXIIRsiRNA could significantly down-regulate MMP2 and MMP9 expression both at mRNA and protein level (Fig. 5A, Fig. 5B), accompanied with which was the increasing of TIMP2 (Fig. 5C). All of the results indicated an important role of AXIIR in degradation of the extracellular matrix. Furthermore, the subsequent effects might result from the inhibition of AKT and ERK pathways (Fig. 5C). Altogether, the results suggested that AXIIRsiRNA might be able to inhibit angiogenesis via inactivating phosphorylation of AKT and ERK1/2 thus reducing MMP2 and MMP9 expression.

In conclusion, we found that AXIIR was expressed in HUVECs and it played a vital role in angiogenesis. Using RNA interference technique to knockdown the expression of AXIIR in HUVECs, we demonstrated that AXIIR was essential in regulating cell proliferation, migration, invasion, tube formation in vitro and angiogenesis in vivo. Furthermore, we proved that knockdown of AXIIR could lead to cell cycle arrest in S/G2 phase while had no affection on cell apoptosis. Moreover, we found that the molecular mechanism might be the inhibition of expression of MMP2 and MMP9. All the data indicate that AXIIR may be a critical regulator in angiogenic responses of ECs.

Disclosure Statement

We declare no conflicts of interest.

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Song et al.: Annexin A2 Receptor siRNA Inhibits Angiogenesis

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