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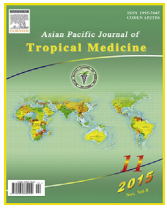

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# Annexin A2 silencing enhances apoptosis of human umbilical vein endothelial cells *in vitro*

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ABSTRACT

**Objective:** To study the effects of inhibited Annexin A2 (ANXA2) on human umbilical vein endothelial cells (HUVECs) *in vitro*.

**Methods:** Short hairpin RNA (shRNA) targeting ANXA2 was designed and cloned into double marked lentivirial vector GV248 for RNAi to generate the recombinant expression plasmids, which were stably transfected into HUVECs. The protein and mRNA expression levels of ANXA2 were analyzed by western blotting and real-time polymerase chain reaction, respectively. Cell proliferation (cell counting kit-8 assay), apoptosis (flow cytometry analysis), the expression (western blotting) and the activity of caspases (enzyme-linked immunosorbent assay) were used to assess the effects of silencing ANXA2 on HUVECs *in vitro*.

**Results:** The plasmids to express ANXA2-specific shRNA were constructed and were infected into HUVEC resulting in the stably transfected experimental (ANXA2-shRNA), control (control-shRNA) and mock (no plasmid) cell lines, which were verified with western blot and real-time PCR. HUVEC/ANXA2-shRNA showed an inhibition rate 91.89% of ANXA2 expression compared to the mock HUVEC. ANXA2 silencing cell strain obviously presented a lower cell proliferation activity compared to the control and mock HUVECs, with an inhibition rate 82.35% on day 7 *in vitro*. FACS analysis indicated that the HUVEC/ANXA2-shRNA cells undergoing apoptosis increased by 102.61% compared to the mock HUVECs ($P < 0.01$). Moreover, the activity levels of caspase-3, caspase-8 and caspase-9 in HUVEC/ANXA2-shRNA cells were increased and the activated cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 were upregulated evidently compared with that of the control and mock HUVECs by 56.29%, 89.59% and 144.58% ($P < 0.01$).

**Conclusions:** shRNA-mediated silencing of ANXA2 could not only be able to suppress HUVECs proliferation but to upregulate the enzyme activity of caspases, which bring to an increase of cell apoptosis. This work suggested that ANXA2 may represent a useful target of future molecular therapies.

## 1. Introduction

Annexin A2 (ANXA2), a kind of calcium depended membrane phospholipids binding protein, is one of the important members of Annexins family which widely expressed in cell membrane, cytoplasm and extracellular matrix [1,2]. ANXA2 can function in many life activities, such as formation of cell membrane structure, membrane fusion, signal transduction, cell migration, DNA synthesis, cell proliferation and cell apoptosis. It is also reported that ANXA2 may play a key role in the retinal neovascularization. With its partner S100A10 (p11), ANXA2 is able to form a heterotetrameric complex [3–10], which binds both plasminogen and its activator (tissue plasminogen activator, tPA) to accelerate the generation of plasmin [11–17]. The isolated endothelial cells from the mice deficient in annexin A2 ($ANXA2^{-/-}$) are unable to support tPA-dependent plasminogen activation *in vitro* [18,19] and the $ANXA2^{-/-}$ mice exhibit reduced angiogenesis in growth factor-stimulated assays in adulthood. Retinal neovascularization is a common pathological change of many eye

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diseases, which results in wide injury to eyes to get blindness [20]. We assumed that ANXA2 may affect the process of neovascularization of the retina, so the objectives of the present study were to focus on the effects of silencing ANXA2 by small hairpin RNA (shRNA) on human umbilical vein endothelial cells (HUVECs) *in vitro* that is usually used in the study of angiogenesis. We found that shRNA-mediated silencing of ANXA2 could not only be able to suppress HUVECs proliferation but to upregulate the enzyme activity of caspases, which bring to an increase of cell apoptosis.

## 2. Materials and methods

### 2.1. Plasmid construction

The shDNAs encoding ANXA2-specific shRNA targeting ANXA2 nucleotides (GeneBank: NM_001002858) (Table 1), which were designed with the free software OPtiRNA (http://optirna.unl.edu/)and synthesized as by GeneChem, China, were used to construct experimental plasmids (pGV248/ANXA2-shRNA) by inserting into an *Age* I and *Eco*R I linearized pGV248, an shRNA shuttle expression vector containing a green fluorescent protein reporter gene (GeneChem, China). A negative control vector (pGV248/control-shRNA) was constructed similarly with an unrelated shRNA sequence that does not suppress the expression of genes expressed in humans (GeneChem). All inserted sequences were verified by DNA sequencing.

### 2.2. Cell culture

HUVEC cells were obtained from ATCC, USA. The cells were cultured in Ham's-F12K medium (Zhongqiaxinzhou Biotechnologies, China), supplemented with 5% fetal calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere with 5% $CO_2$ at 37 °C.

### 2.3. Cell transfection

For experimentation, HUVEC cells were *in vitro* seeded into 24-well plates, allowed to grow to 80%–90% confluence, and transfected by plasmids (pGV248/control-shRNA and pGV248/ANXA2-shRNA) with DNA Transfection Reagent (Invitrogen, USA) according to the manufacturer's instruction. At 48 h after transfection, cells were selected by culturing in the presence of 400 μg/mL of G418 (Sangon Biotech, China) for 2 weeks. Individual G418-resistant monoclonals were obtained by performing a limiting dilution with subsequent proliferation in medium supplemented with 200 μg/mL of G418 to generate the stably transfected experimental (HUVEC/ANXA2-shRNA), control (HUVEC/control-shRNA) and mock (no plasmid) cell lines. The efficiency of infection was monitored by fluorescence microscopy.

### 2.4. Real-time PCR analysis

Total RNA was isolated from cells using an RNeasy kit (Invitrogen, USA). Single-stranded cDNA was synthesized from 1 μg total RNA using an oligo (dT) 18-mer as primer, and reverse transcription (Invitrogen, USA) in a final reaction volume of 25 μL. Quantitative real-time PCR was performed using the TaqMan method and Probe Real Master Mix (Tiangen Biotech, Beijing, China) in a 7 500 real-time PCR System (Applied Biosystems Inc, CA). *GAPDH* was used as control. The following primers were used: i) *ANXA2* forward, 5′-GTGAAGAGGAAAGGAACCGA-3′ and reverse, 5′-CTTGATGCTCTCCAGCATGT-3'; and ii) *GAPDH* forward, 5′-GCCTTCCGTGTTCCTACC-3′ and reverse, 5′-AGAGTGGGAGTTGCTGTTG-3'. The analysis was performed using the $2^{-\Delta\Delta Ct}$ method and the relative value of *ANXA2* mRNA expression was normalized to *GAPDH* gene expression.

### 2.5. Western blot analysis

Total protein was isolated from cultured cells with the use of an extraction kit (Sangon Biotech, China) according to the manufacturer's instructions. Each 20 mg of protein was separated by 15% SDS-PAGE and then transferred onto PVDF membranes and blocked with 3% BSA in Tris-buffer. Mouse anti-human monoclonal antibody (Cell Signaling Technology, USA) was used. Detection was performed with HRP-conjugated goat anti-mouse immunoglobulins and enhanced chemiluminescence (Clinx, China). Bands were subsequently visualised and analyzed by a ChemiScope5600 integrated chemiluminescence imaging system (Clinx, China). ANXA2 levels were presented as relative ratio (RR) and calculated using the formula for signal intensity (SI) of ANXA2 and GAPDH: $RR = SI_{ANXA2}/SI_{GAPDH}$.

### 2.6. Cell proliferation assay

Cell proliferation was evaluated using a cell counting kit-8 (Beyotime Institute of Biotechnology, China). Cells and blank controls were seeded in 96-well plates ($2 \times 10^3$ cells/well with 100 μL medium) and for 24 h. Next, 10 μL CCK-8 solution was added to the culture medium for 2 h and the absorbance ($A_{490}$) was recorded by a microplate reader (BioTek, USA). This was repeated 3 times at various time points.

### 2.7. Enzyme-linked immunosorbent assay

HUVEC caspase-8, caspase-9 and caspase-3 levels were estimated with the use of enzyme-linked immunosorbent assay kits (Innovative Research, USA) according to the manufacturer's instructions. Colorimetric analysis was performed on a microplate reader (Bio-Rad Laboratories, USA) and the absorbance

**Table 1**
Synthesized shDNAs encoding ANXA2-specific shRNA.

| ID | 5′ | Stem | Loop | Stem | 3′ |
|---|---|---|---|---|---|
| ANXA2 | ccgg | CGGGATGCTTTGAACATTGAA | CTCGAG | TTCAATGTTCAAAGCATCCCG | TTTTTg |
| | aattcaaaaa | CGGGATGCTTTGAACATTGAA | CTCGAG | TTCAATGTTCAAAGCATCCCG | |
| Control | ccgg | CTACCGTTGTTAAGGTGT | CTCGAG | GACACCTATAACAACGGTAG | TTTTTg |
| | aattcaaaaa | CTACCGTTGTTAAGGTGT | CTCGAG | GACACCTATAACAACGGTAG | |

($A_{405}$) of the samples was measured to calculate the protein concentrations according to the standard curve generated.

### 2.8. FCM analysis of cell apoptosis

HUVEC/ANXA2-shRNA, HUVEC/control-shRNA and the mock HUVECs were stained relatively with FITC-conjugated annexin V and propidium iodide (PI) as supplied by an apoptosis detection kit (Beyotime, China). Cells were analyzed using a FACSCalibur (BD Biosciences, USA).

### 2.9. Statistical analysis

Statistical significance was assessed by the 2-tailed Student *t* test. *P* values of <0.05 were considered statistically different.

## 3. Results

### 3.1. ANXA2 expression in HUVEC cells is inhibited by shRNA in vitro

The silencing efficiency of ANXA2-specific shRNA in HUVEC cells reached approximately 82%. As shown in Figure 1b, the ratio of ANXA2 to GAPDH demonstrated that the expression levels of ANXA2 protein were significantly lower in the HUVEC/ANXA2-shRNA cells than in the HUVEC/control-shRNA cells and in the mock HUVECs ($P < 0.01$). The levels detected in the HUVEC/control-shRNA cells and HUVECs were not significantly different ($P > 0.05$). It was suggested that the *ANXA2* mRNA was markedly downregulated following transfection with stably expressing shRNA at the protein level.

### 3.2. ANXA2 mRNA expression after RNA interference in vitro

Quantitative PCR showed that the expression of *ANXA2* mRNA in HUVECs transfected with shANXA2 was significantly inhibited (Figure 2), and the reduction of the *ANXA2* mRNA expressions was 85% down ($P < 0.01$). The mRNA relative levels were measured in sample rate/cutoff rate (S/CO).

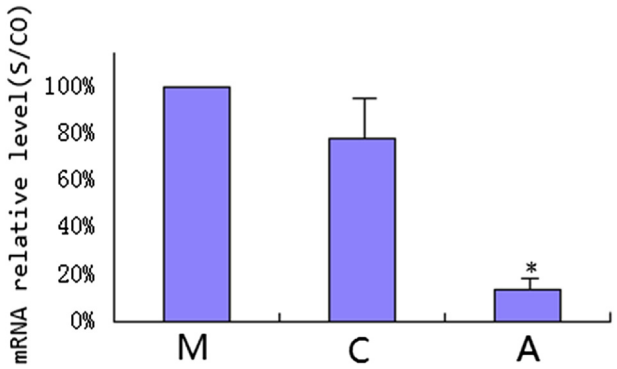


**Figure 2.** Real-time PCR of *ANXA2* in HUVECs with siANXA2 transfection immediately.
M, mock HUVEC; C, HUVEC/control-shRNA; A, HUVEC/ANXA2-shRNA.

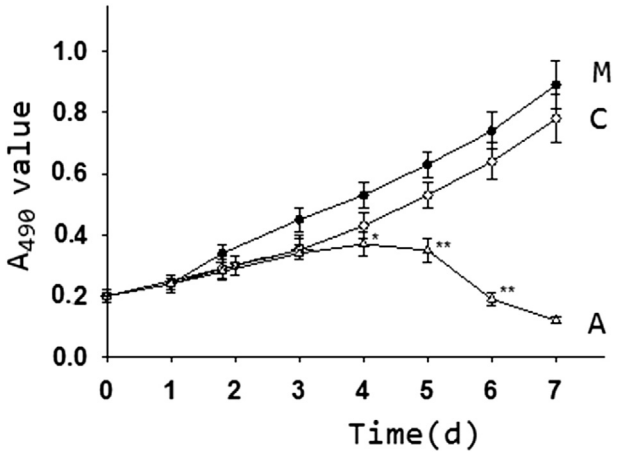


**Figure 3.** Cell proliferation assay.
$^{*}P < 0.05$, $^{**}P < 0.01$ vs. mock HUVEC. M, mock HUVEC; C, HUVEC/control-shRNA; A, HUVEC/ANXA2-shRNA.

### 3.3. Suppression of HUVEC proliferation

The effect of ANXA2 suppression on the proliferation following transfection with specific shRNA is shown in Figure 3. At 72 h after transfection, the cell proliferation ability

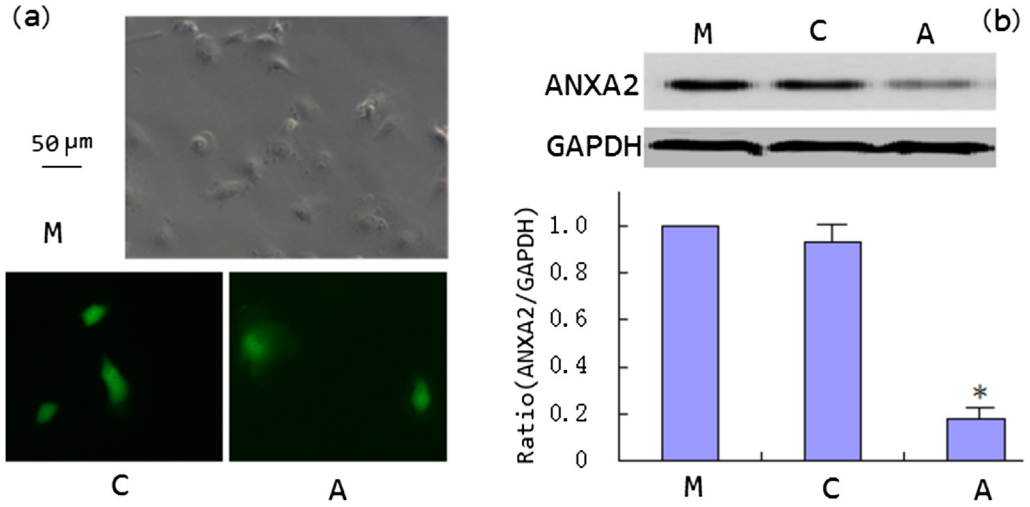


**Figure 1.** ANXA2 expression level in HUVECs and silencing efficiency of shRNA.
(a): Representative immunofluorescence images. (b): Representative Western blotting image of ANXA2 silencing upon transfection of shRNA. $^{*}P < 0.01$ *vs.* mock HUVEC. M, mock HUVEC; C, HUVEC-control-shRNA; A, HUVEC-ANXA2-shRNA.

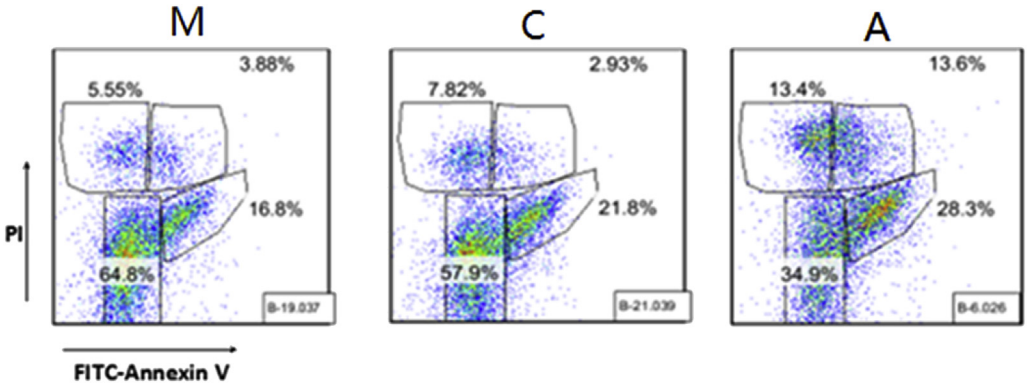


**Figure 4.** FACS analysis of HUVEC apoptosis.
M, mock HUVEC; C, HUVEC/control-shRNA; A, HUVEC/ANXA2-shRNA. Right lower quadrant: early apoptotic cells; Left upper quadrant: dead cells; Left lower quadrant: live cells; Right upper quadrant: late apoptotic cells. All apoptosis experiments were carried out more than twice. Results were similar and one representative experiment is shown.

**Table 2**
Activity levels of caspase-3, -8 and -9 in HUVECs.

| Groups | Caspase-3 activity | Caspase-8 activity | Caspase-9 activity |
|---|---|---|---|
| mock HUVEC | 0.063 ± 0.002 | 0.021 ± 0.003 | 0.035 ± 0.005 |
| HUVEC/control-shRNA | 0.070 ± 0.004 | 0.038 ± 0.003 | 0.049 ± 0.003 |
| HUVEC/ANXA2-shRNA | 0.112 ± 0.003[a] | 0.061 ± 0.007[a] | 0.053 ± 0.008[a] |

[a] *vs.* mock HUVEC, $P < 0.01$.

in HUVEC/ANXA2-shRNA cells was significantly decreased ($P < 0.01$ or $P < 0.05$) compared with that in the HUVEC/control-shRNA cells and in the mock HUVECs. The result indicated that the growth of HUVEC/ANXA2-shRNA cells was markedly inhibited *in vitro*.

### *3.4. Silencing ANXA2 enhances apoptosis of HUVECs*

Cells were analyzed using an FACS by the staining method with FITC-conjugated annexin V and PI (Figure 4). The number of HUVEC/ANXA2-shRNA cells undergoing apoptosis was 41.9% and increased by 102.61% compared to the mock HUVECs ($P < 0.01$), which suggests that ANXA2 silencing enhances apoptosis in HUVECs.

### *3.5. Silencing ANXA2 affects the levels of caspase-3, -8 and -9*

Since the above results suggest that silencing of ANXA2 upregulates apoptosis, we intended to investigate the activity of apoptosis-related caspases. The activity levels of HUVEC caspase-3, -8 and -9 were estimated with the use of ELISA kits (Table 2). The levels of caspase-3, -8 and -9 in HUVEC/ANXA2-shRNA cells was 0.112, 0.061 and 0.053, respectively compared with 0.063, 0.021 and 0.035 in the mock HUVECs ($P < 0.01$). It suggests that ANXA2 silencing increased the activity levels of HUVEC caspase-3, caspase-8 and caspase-9. Furthermore, the total protein was isolated from cultured cells and was analyzed by means of western blotting (Figure 5). The cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 were upregulated by 56.29%, 89.59% and 144.58% compared to that in mock HUVECs ($P < 0.01$).

## 4. Discussion

ANXA2 is a member of the annexin family of proteins and exists either in monomeric form or as a heterotetramer containing two light chains of S100A10/p11 and two chains of ANXA2, which has recently drawn attention for its ability to regulate multiple key processes in cells [21–23]. In the present study, we focus on the effects of silencing ANXA2 by

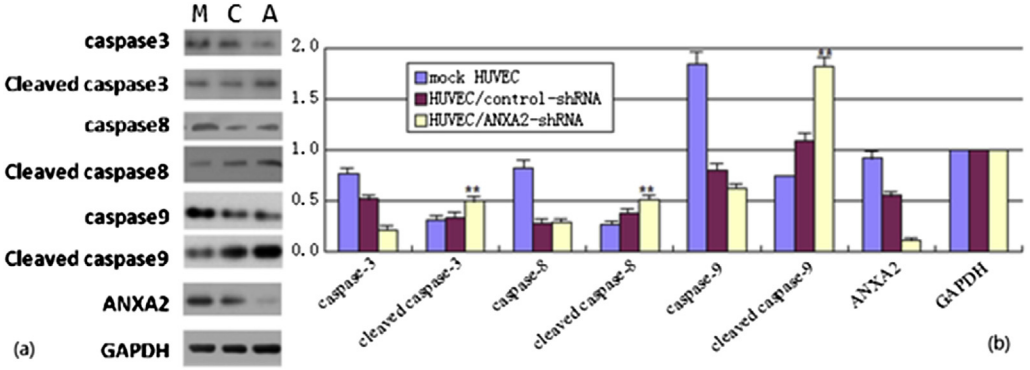


**Figure 5.** Western blot analysis.
M, mock HUVEC; C, HUVEC/control-shRNA; A, HUVEC/ANXA2-shRNA.

shRNA on human umbilical vein endothelial cells (HUVECs) *in vitro*.

shRNA is a sequence of RNA that makes a tight hairpin turn that can be used to silence target gene expression via RNA interference (RNAi) [24,25]. Expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors [26]. In this work, the shRNA targeting ANXA2 was designed and cloned into double marked lentivirial vector GV248 for RNAi to generate the recombinant expression plasmids, which were infected into HUVEC resulting in the stably transfected experimental (ANXA2-shRNA) cell line, HUVEC/ANXA2-shRNA, with an inhibition rate 91.89% of ANXA2 expression compared to the mock and the control HUVECs.

Our data showed that ANXA2 silencing cell strain obviously presented a lower cell proliferation activity compared to the control and mock HUVECs. In addition, FACS analysis indicated that the HUVEC/ANXA2-shRNA cells undergoing apoptosis increased by 102.61% compared to the mock HUVECs ($P < 0.01$), which is in agreement with previous research. How ANXA2 affects cell apoptosis? Huang [27] reported that ANXA2 was involved in P53-mediated apoptosis in that ANXA2 levels decreased significantly in P53 induced cell apoptosis. Madureira *et al* [28–31] revealed that ANXA2 could protect DNA from degradation. These results suggested that ANXA2 may affect the P53 expression and its activity, and the down proteins such as Bcl2, Bax, and caspase family to inhibit cell apoptosis. In this paper, we have also confirmed this viewpoint. The activated cleaved caspase-3, cleaved caspase-8 and cleaved caspase-9 of the HUVEC/ANXA2-shRNA cells were upregulated evidently compared with that of the control and mock HUVECs by 56.29%, 89.59% and 144.58% ($P < 0.01$).

Collectively, the results revealed a link between the level of ANXA2 expression and cell apoptosis, which suggested that the potential utility of ANXA2 as a predictive biomarker for detecting angiogenesis and that ANXA2 might be used in the treatment of neovascularization disorders as a therapeutic target of molecular-based strategies.

## Conflict of interest statement

We declare that we have no conflict of interest.

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