Overexpression of HTRA1 increases the proliferation and migration of retinal pigment epithelium cells

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Abstract

Background. Age-related macular degeneration (AMD) mainly affects the central region of retina and has many late-stage manifestations.

Objectives. Age-related macular degeneration is a leading cause of irreversible blindness in older people. The main feature of AMD is retinal pigment epithelium (RPE) degeneration. In this study, we aimed to explore the influence of HTRA1 expression on the proliferation and migration of RPE cells.

Materials and methods. Human ARPE-19 cells were transfected with an HTRA1 overexpression lentivirus or HTRA1 siRNA to silence HtrA1 expression. Quantitave reverse-transcription polymerase chain reaction (qRT-PCR) and western blotting were used to verify the relative level of HTRA1 mRNA and expression of HTRA1 protein of transfected human ARPE-19 cells. The MTT clone formation and transwell assays were used to confirm the effect of HTRA1 expression on the proliferation, colony forming ability and migration of ARPE-19 cells.

Results. The proliferation capacity (shown as optical density value) of ARPE-19 cells in the HTRA1-overexpressing group at culture times of 24 h and 48 h were 0.595 ±0.032 and 0.867 ±0.037 respectively, which were much higher than in the mock group. However, the proliferative capacity of cells in the HTRA1-silenced group decreased with increasing time of culture, compared with the mock group. The number of cloned and migrating cells in the HTRA1-overexpressing group were much higher than in the mock group, whereas the numbers in the HTRA1-silenced group were significantly lower.

Conclusions. Overexpression of HTRA1 promotes proliferation and migration of RPE cells, which can help maintain the function of sensory neurons in the retina. Therefore, HTRA1 may be a suitable target for AMD treatments.

Key words: proliferation, migration, HtrA1, AMD, RPE

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Background

Age-related macular degeneration (AMD) is an eye disease that mainly affects the central region of retina with many late-stage manifestations.[1] An abundance of cone photoreceptors in the retina can impact visual acuity. Previous studies have reported pathological changes during the development of AMD. However, the pathogenesis of AMD still needs to be explored further.[2] Retinal pigment epithelium (RPE) degeneration is one of the most important characteristics of AMD and plays a key role in regulating the neurosensory retina. The unusual structure of the RPE is closely related to the etiology of AMD.[3,4] Previous data have shown that age-related maculopathy susceptibility 2 (ARMS2) and HtrA serine peptidase 1 (HTRA1) located at chromosome 10q26 are closely associated with susceptibility to AMD.[5,6] Wang et al. speculated that HTRA1 might be an important risk factor in AMD.[7]

Human HTRA1 is a member of the serine protease family, which regulates protein quality and cell fate.[8] Aberrant expression of HTRA1 was found in a variety of tumors. Some studies have shown that overexpression of HTRA1 restrains tumor growth, indicating that HTRA1 might be a tumor-inhibiting factor.[9,10] HTRA1 may regulate the progression of AMD by mediating a variety of different pathways. Various substrates of HTRA1 have been identified, such as fibronectin, aggrecan and the transforming growth factor-beta (TGF-β) family.[11] Moreover, HTRA1 was shown to inhibit signaling via the TGF-β family.

Objectives

In this study, RPE cells were transfected with an HTRA1 overexpression lentivirus or HTRA1 siRNA. The effects of HTRA1 on the proliferation and migration of RPE cells were investigated.

Materials and methods

Cell culture

The human retinal pigment epithelial cell line ARPE-19 was purchased from Cell Repository, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA), and 100 mg/L streptomycin and $1*10^{-5}$/UI penicillin (Gibco) at 37°C/5% CO_2.

Establishment of HTRA1-overexpressing cells

A pcDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, USA) was used to establish stably transfected HTRA1-overexpressing cells. To construct the pcDNA3-HTRA1 plasmid, the full-length human HTRA1 gene *pB4* was digested with EcoRI and inserted into an EcoRI-cleaved pcDNA3 vector. ARPE-19 cells were transfected with pcDNA3 or pcDNA3-HTRA1 using the lipofection technique according to the manufacturer's protocol (Gibco BRL, Life Technologies, Rockville, USA).

Transfection of siRNAs

Human HTRA1 siRNA and scrambled control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). ARPE-19 cells were seeded onto multiple-well plates in DMEM containing 10% FBS and placed in a humidified incubator at 37°C and 5% CO_2. The cells were then transfected with 80 nM HTRA1 or non-target (control) siRNAs for 72 h using 2 μL/mL Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions.

Cell proliferation assay

Cells were seeded into 96-well plates at a density of 2×10^3 cells/well. The CCK-8 assay was used to indirectly determine cell growth.

qRT-PCR

Total RNA was isolated using the Total RNA Isolation System (Promega, Madison, USA). The cDNA was generated from 1 ug total RNA per sample using anchored oligo-dT primers (Reverse-iT First Strand Synthesis; ABgene, Thermo Fisher Scientific, Waltham, USA). Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using a LightCycler and FastStart DNA Master SYBR Green 1 kit (Roche Applied Sciences, Basel, Switzerland).

Western blotting

Protein samples were extracted from cells and fractionated using 7.5–10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). A primary antibody against HTRA1 (Stressgen Bioreagents, Ann Arbor, USA) was used, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Research Diagnostics, Concord, USA) as an internal control.

Clone formation assay

Cells (100/well) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS in six-well plates for 14 days. The number of clones (≥50 cells) was counted under a microscope.

Transwell assay

The transwell migration assay was performed in chemotaxis chambers containing 24 wells. A total of 5×10^4

cells were inoculated into the top chamber in DMEM (200 µL) without serum. The bottom chambers contained DMEM (600 µL) supplemented with 10% FBS. Cells that migrated through the pores to the bottom chamber were fixed in paraformaldehyde (4%) and stained with crystal violet. The number of cells was counted using a microscope (model DM4000B; Leica, Wetzlar, Germany).

Statistical analyses

One-way analysis of variance (ANOVA) and paired Student's t-tests were used and IBM SPSS v. 20.0 software (IBM Corp., Armonk, USA) was utilized. Significance level was defined as $p < 0.05$.

Results

Verification of transfection efficiency

Sequences of the constructed vector expressing HTRA1 (Fig. 1A) and HTRA1-shRNA (Fig. 1B) are shown. The HTRA1 mRNA and protein expression levels in each group were detected with qRT-PCR and western blot, respectively. The relative HTRA1 mRNA level in the HTRA1-overexpressed group was 0.291 ±0.035, which was significantly higher than in the mock group (0.075 ±0.014, $p < 0.01$). The HTRA1 mRNA expression in the sh-HTRA1#1 and sh-HTRA1#2 groups were 0.027 ±0.008 and 0.017 ±0.012, respectively, which were markedly lower than in the mock group ($p < 0.01$) and sh-Scb group (0.087 ±0.010) (Fig. 2A). The HTRA1 protein expression in the HTRA1-overexpressing group was much higher than in the mock group, indicating that the lentivirus transfection was successful (Fig. 2B). The HTRA1 protein level in the shRNA-HTRA1 groups were dramatically lower in comparison to the mock and sh-Scb groups ($p < 0.01$), suggesting that HTRA1 was knocked down by siRNA transfection.

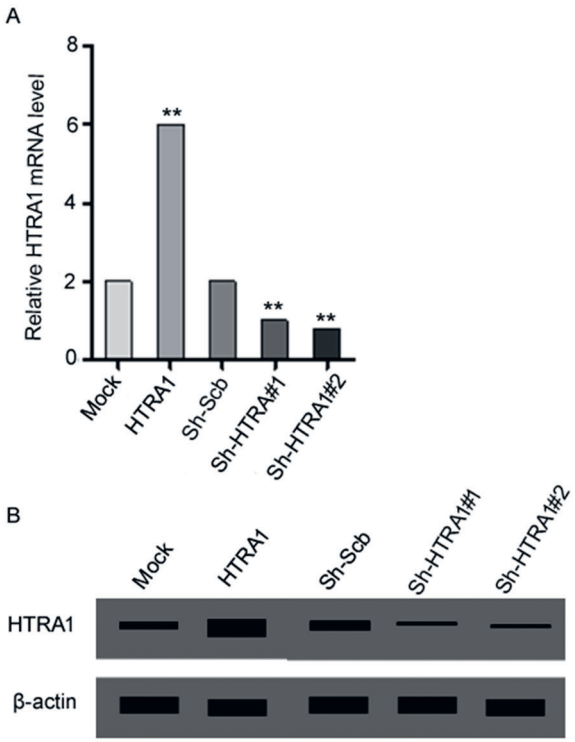

Fig. 2. Effect of plasmid or lentivirus transfection on HTRA1 expression. A. Relative HTRA1 mRNA level after transfection with HTRA1 overexpression lentivirus or HTRA1 siRNA plasmid (**$p < 0.01$ compared to mock group); B. Expression of HTRA1 protein in mock, HTRA1, shRNA-scb, and shRNA-HTRA1 groups

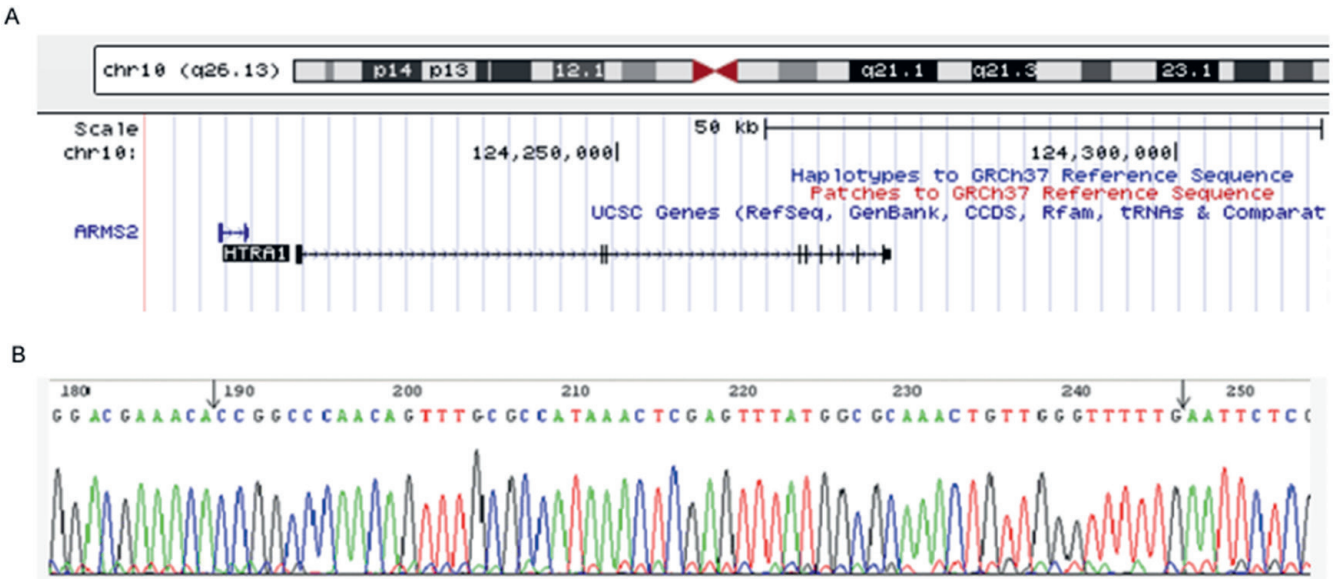

Fig. 1. Design of HTRA1 overexpression and silencing vectors. A. Constructed HTRA1 overexpression lentivirus; B. Constructed HTRA1-shRNA sequence

HTRA1 overexpression promotes proliferation of ARPE-19 cells

To investigate the effect of HTRA1 on the proliferation of ARPE-19 cells, CCK-8 was used to determine the proliferative capacity in all groups. As shown in Fig. 3, the proliferative capacity of ARPE-19 cells at 0 h in the mock, HTRA1-overexpressing (0.255 ±0.014), Sh-Scb (0.257 ±0.013), sh-HTRA1#1 (0.257 ±0.012), and sh-HTRA1#2 (0.257 ±0.016) groups were almost the same. As the culture time increased, the proliferative capacity of ARPE-19 cells in the HTRA1-overexpressing group significantly increased, compared with the mock group. At 24 h and 48 h, the optical density (OD) values in the HTRA1 group were 0.595 ±0.032 and 0.867 ±0.037, respectively, which were much higher than those in the mock group (0.460 ±0.028 and 0.646 ±0.035, respectively, $p < 0.01$). At 24 h and 48 h, the OD values in sh-HTRA1#1 group were 0.426 ±0.051 and 0.614 ±0.042, respectively, and 0.383 ±0.061 and 0.537 ±0.058, respectively, in the sh-HTRA1#2 group. The OD values in the sh-HTRA1#1 and sh-HTRA1#2 groups were significantly lower compared to the mock group at both time points ($p < 0.01$).

Overexpression of HTRA1 in ARPE-19 cells promotes invasion and vascular tube formation

To further explore the role of HTRA1 on AMD, we performed clone formation and transwell chamber analyses to assess the effect of HTRA1 overexpression or silencing on the cloning formation and migration of ARPE-19 cells. Compared with the mock group, the HTRA1-overexpressing group showed much more cloning cells in the clone formation assay, whereas the number of cells in the shRNA-HTRA1 groups were significantly lower than that in the mock and shRNA-scramble groups (Fig. 4A). These findings indicate that overexpression of HTRA1 in ARPE-19 cells promotes colony formation. The transwell migration assay results show that the number of migrating cells in the HTRA1-overexpressing group was much higher compared to the mock group, while the migration capacity of ARPE-19 cells in the shRNA-HTRA1 groups were lower compared to the mock and shRNA-scramble groups (Fig. 4B).

Discussion

The AMD is a major cause of irreversible blindness in the elderly population.[12] The key feature of AMD

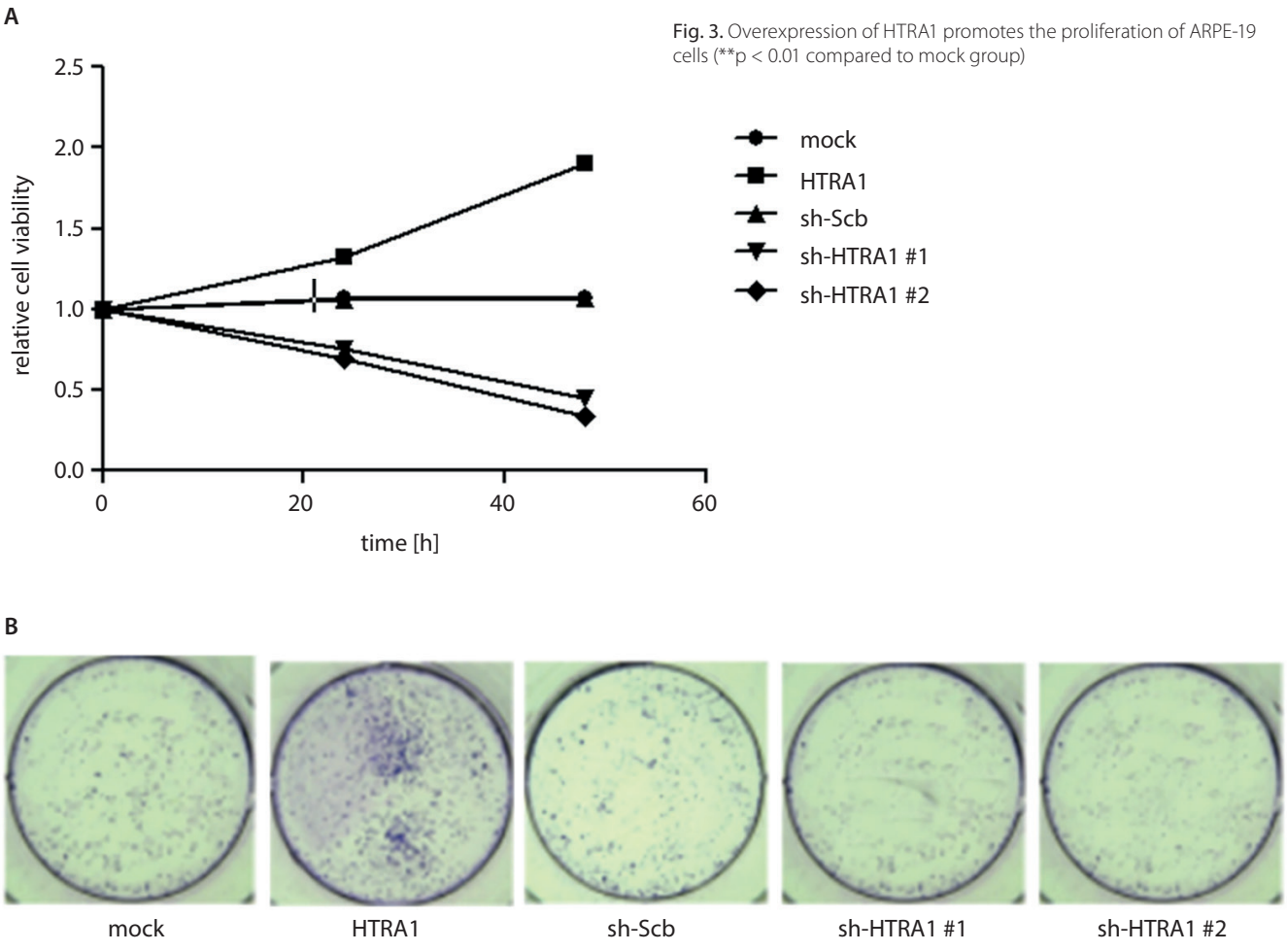

Fig. 3. Overexpression of HTRA1 promotes the proliferation of ARPE-19 cells (**$p < 0.01$ compared to mock group)

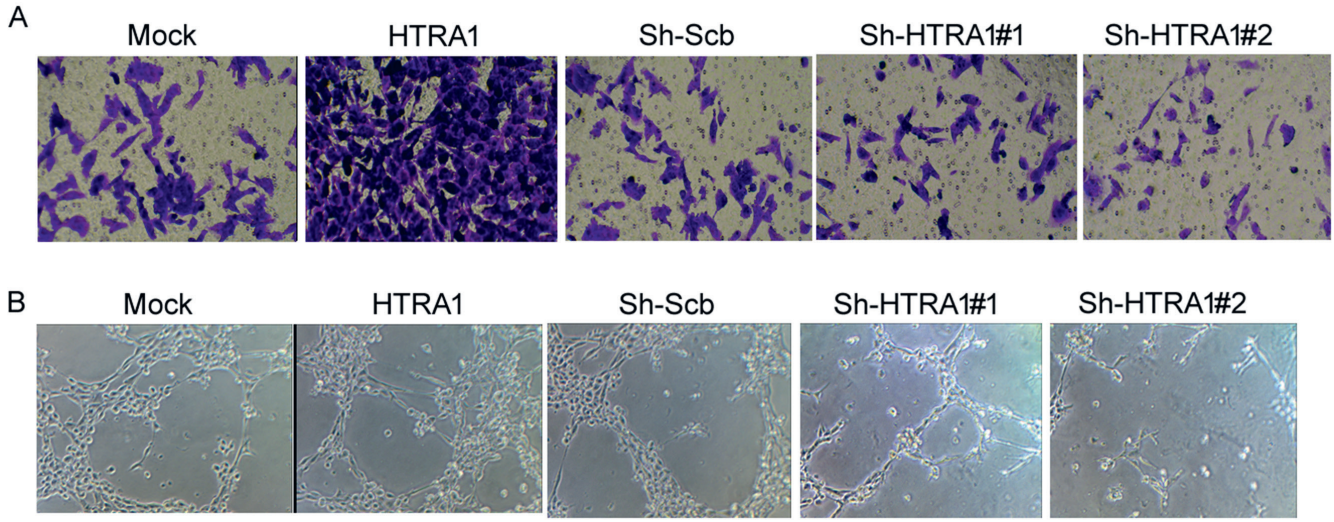

Fig. 4. Overexpression of HTRA1 promotes colony formation ability and migration of ARPE-19 cells. A. Colony formation in mock, HTRA1, shRNA-scb, and shRNA-HTRA1 groups; B. Migration of ARPE-19 cells in mock, HTRA1, shRNA-scb, and shRNA-HTRA1 groups

is degeneration of the RPE, which is located between retinal photoreceptors and choroidal capillaries. Dysfunction of RPE cells can destroy photoreceptors and disrupt the choroid vascular system.[13] In AMD, focal extracellular deposits in the Bruch's membrane are identified as drusen in ophthalmic testing. The presence of drusen and dense deposits are often considered to indicate the early stages of AMD.[14] The 2 key factors associated with the development of AMD are ARMS2 and HTRA1.[15] Specific HTRA1 alleles are related to neovascular lesion extent.[16] Increased expression of HTRA1 in the RPE of mice promoted the exudative form of AMD.[17] HTRA1 is a serine protease that controls protein quality and cell fate.[18] Previous studies have reported that HTRA1 might affect the occurrence and development of AMD by regulating extracellular matrix (ECM) proteoglycan degradation and TGF-β family activity.[19,20]

In this study, we explored the role of HTRA1 in ARPE-19 cells. The expression of HTRA1 in was increased or decreased by transfecting ARPE-19 cells with an HTRA1 overexpression plasmid or shRNA against HTRA1, respectively. Cell migration is an important part of the immune response and also plays a role in the development of diseases, including inflammation and tumor metastasis.[21] Indeed, cell migration is a key event of many physiological phenomena.[22] Cell viability, migration, and invasion are highly involved in cancer pathogenesis and other biological processes.[23] Control of cell proliferation is a fundamental aspect of tissue formation and regeneration.[24] To investigate the effect of HTRA1 on AMD, we assessed the proliferation, cloning formation, migration and angiogenic ability of ARPE-19 cells with overexpression or silencing of HTRA1. Our data show that overexpression of HTRA1 promotes proliferation, migration, cloning formation, and angiogenic ability of ARPE-19 cells, whereas HTRA1 silencing inhibits all these abilities.

Conclusions

Overexpression or silencing of HTRA1 affects the development of AMD. HTRA1 overexpression in ARPE-19 cells promoted proliferation, migration and vascular tube formation.

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