

E-cadherin involvement in human lens epithelial cell transdifferentiation may be associated with N-cadherin

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Abstract. E-cadherin, β -catenin and N-cadherin serve key roles in the epithelial-to-mesenchymal transition (EMT) that leads to human lens epithelial cell (LEC) transdifferentiation and subsequent cataract formation. The present study aimed to investigate the role of E-cadherin in LEC transdifferentiation. SRA01/04 human LECs were transfected with E-cadherin short interfering (si)RNA (E-cadherin siRNA group), negative control siRNA (NC group) or the transfection reagent Lipofectamine 2000 (blank group). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect mRNA expression levels of E-cadherin, N-cadherin and β -catenin, and western blot analysis was performed to measure the protein expression levels in the three groups. SRA01/04 cells transfected with E-cadherin siRNA exhibited a significant decrease in the mRNA expression level of E-cadherin ($P<0.05$) and N-cadherin ($P<0.05$), whereas no significant changes were identified for β -catenin expression ($P>0.05$). Consistent with the results of RT-qPCR, western blotting demonstrated that the protein expression levels of E-cadherin and N-cadherin were notably decreased in E-cadherin siRNA-transfected cells, whereas the protein expression level of β -catenin remained unchanged. Results from the present study indicated that E-cadherin may be involved in human LEC transdifferentiation by affecting N-cadherin expression.

Introduction

Cataracts affect ~25 million people worldwide and is the most common cause of blindness (1). Previous studies have reported that lens epithelial cells (LECs) may be induced by

transforming growth factor- β (TGF- β) to undergo epithelial-to-mesenchymal transition (EMT), which may result in cataract formation (2,3). EMT is characterized by the loss of epithelial marker expression (such as E-cadherin) and the gain in expression of mesenchymal markers (including vimentin and fibronectin) (4).

As a member of the classical cadherin family, epithelial (E)-cadherin is a calcium-dependent adhesion molecule that mediates cell-cell adhesion in a number of tissues in different species (5). The inhibition of E-cadherin and the suppression of glycogen synthase kinase-3 β (GSK-3 β) activity mediated by integrin-linked kinase 1 (ILK-1) have previously been reported to be involved in EMT (6-8); E-cadherin may recruit β -catenin to form complexes that are also involved in EMT (9). As a member of an adhesion complex, β -catenin serves a pivotal role in linking the actin cytoskeleton with the cadherin-mediated cell-cell contacts in the cytoplasm (10), and the noncanonical β -catenin signaling pathway has been considered crucial for mediating EMT (11). In addition, it has been demonstrated that decreased E-cadherin expression may lead to the release of β -catenin into the cytoplasm, which may migrate into the nucleus and activate the expression of certain transcription factors that induce EMT (12). Neural (N)-cadherin also belongs to the classical cadherin superfamily and mediates calcium-dependent cell-cell adhesion (13); N-cadherin has been referred as a mesenchymal cadherin that is associated with EMT (14). A previous study demonstrated that N-cadherin protein expression and β -catenin nuclear translocation may be repressed by injury of cultured retinal pigment epithelial cells treated with peroxide (15). Another report revealed that the gain of N-cadherin expression and the loss of E-cadherin expression (known as 'cadherin switching') is a major hallmark of EMT (16). However, it is unknown whether there is a transcriptional regulatory relationship between E-cadherin, β -catenin and N-cadherin.

In the present study, E-cadherin short interfering RNA (siRNA) transfection was used to knock down the expression of E-cadherin in the SRA01/04 human lens epithelial cell (LEC) line. Subsequently, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were used to investigate the effects on mRNA and protein expression levels, respectively, of E-cadherin, N-cadherin and β -catenin to examine the role of E-cadherin in EMT of human LECs.

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Materials and methods

Culture of human LEC line SRA01/04. The SRA01/04 human LEC line was purchased from Shanghai SXBIO Biotechnology Co., Ltd. (Shanghai, China). SRA01/04 cells were cultured in the Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; 1:1) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified 5% CO₂ incubator at 37°C. SRA01/04 cells were grown to 80–90% confluence, digested with a 0.25% trypsin-0.02% ethylenediamine-N,N,N',N'-tetraacetic acid solution (Gibco; Thermo Fisher Scientific, Inc.), centrifuged at 100 x g for 5 min at room temperature and transferred into a new culture dish for passage. SRA01/04 cells were stored in frozen stock solution at -80°C containing 10% dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 40% FBS and 50% DMEM/F12.

E-cadherin siRNA transfection. Human E-cadherin siRNA and negative control (NC) siRNA was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: E-cadherin siRNA forward, 5'-GAGGCUGUAUACACCAUAUTT-3' and reverse, 5'-AUAUGGUGUAUACAGCCUUTT-3'; NC siRNA forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGAGAATT-3'. SRA01/04 cells (3x10⁵) were seeded into a 6-well plate with 2 ml antibiotic-free DMEM/F12 medium 24 h prior to transfection at 37°C. Healthy, well-adhered 70–90% confluent cells were used for transfection. E-cadherin and NC siRNAs were diluted in 250 µl Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.) to make a concentration of 20 µM stock solution; 3.3 µl stock solution was subsequently added to a 6-well plate to a final concentration of 66 nM and mixed gently 3–5 times. Lipofectamine 2000 transfection reagent (10 µl; Invitrogen; Thermo Fisher Scientific, Inc.) was diluted in 250 µl Opti-MEM, mixed and incubated for 5 min at room temperature. The diluted siRNA and transfection reagent were combined and incubated at room temperature for 20 min. Subsequently, 500 µl of the mixture was added to each well of the 6-well plate and mixed. Cells were transfected with the E-cadherin siRNA (siRNA group), the NC siRNA (NC group) or with Lipofectamine 2000 (blank group) and were cultured at 37°C in a 5% CO₂ incubator for 48 h; the medium was replaced with fresh antibiotic-free medium following 12 h of transfection.

Total RNA isolation and RT-qPCR analysis. Total RNA was extracted from 1x10⁶ cultivated cells using the RNAiso Plus kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Briefly, cells were lysed in 1 ml RNAiso Plus for 5 min at room temperature. The lysate was centrifuged at 12,000 x g for 5 min at 4°C, and the aqueous phase containing RNA was separated from the organic phase via incubation with 200 µl chloroform for 5 min at room temperature. The solution was subsequently centrifuged at 12,000 x g for 15 min at 4°C, and the supernatant was transferred into a new tube and incubated with 700 µl isopropanol for 10 min at room temperature. RNA was precipitated via centrifugation at 12,000 x g for 10 min at 4°C. The RNA pellet was washed with 75% ethanol,

Table I. Sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'→3')
E-cadherin	F: GAGAACGCATTGCCACATACA R: ACCTTCCATGACAGACCCCTTAA
N-cadherin	F: TCTCTCAGCTGTGTTCATCCAAC R: CACAGAGGTTCCTGGAAGAGCAC
β-catenin	F: CAATGACTCGAGCTCAGAGGGTAC R: TTTAGCAGTTTTGTTCAGTTCAGGGA
GAPDH	F: AATCCCATCACCATCTTCCAG R: ATCAGCAGAGGGGGCAGAGA

E, epithelial; N, neural; F, forward; R, reverse.

air dried for 5–10 min and redissolved in 30 µl diethyl pyrocarbonate (DEPC)-treated water. To assess the quality of RNA, A260/A280 and A260/A230 ratios of the RNA samples were measured using an SMA4000 Ultraviolet spectrophotometer (Merinton Instruments, Ltd., Beijing, China). An A260/A280 ratio between 2.0 and 2.3 was considered high quality RNA (17) and was used for the subsequent experiments.

cDNA was synthesized using a PrimeScript RT-PCR kit (Takara Bio, Inc.) according to the manufacturer's protocol. Primers for E-cadherin, N-cadherin, β-catenin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China; Table I). qPCR reactions were set up as follows: 10 µl SYBR Premix Ex Taq (2X; Takara Bio, Inc.), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 2 µl cDNA (adjusted to the same concentration) and 6 µl double-distilled H₂O. qPCR was performed using a ViiA 7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following conditions: 50.0°C for 3 min and 95.0°C for 3 min; followed by 40 cycles of 95.0°C for 10 sec and 60.0°C for 30 sec; followed by melting curve construction and analysis. GAPDH was used as a reference gene. Each sample was repeated three times, and calculation of gene expression was performed by the 2^{-ΔΔC_q} method (18).

Protein extraction and western blot analysis. A total of 5x10⁶ SRA01/04 cells were washed three times with pre-cooled phosphate-buffered saline (Gibco, Thermo Fisher Scientific, Inc.) and lysed at 4°C for 30 min with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1% phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA). The supernatant without cell debris was obtained by centrifugation at 19,000 x g for 10 min at 4°C. Protein concentration was determined by Bicinchoninic Acid Protein Assay kit (Sangon Biotech, Co., Ltd., Shanghai, China) according to the manufacturer's instruments.

A total of 30 µg protein was loaded in each well and separated by 10% (w/v) SDS-PAGE (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were transferred onto a polyvinylidene fluoride membrane (Merck KGaA) and blocked with 5% non-fat dry milk for 1 h at room temperature. The

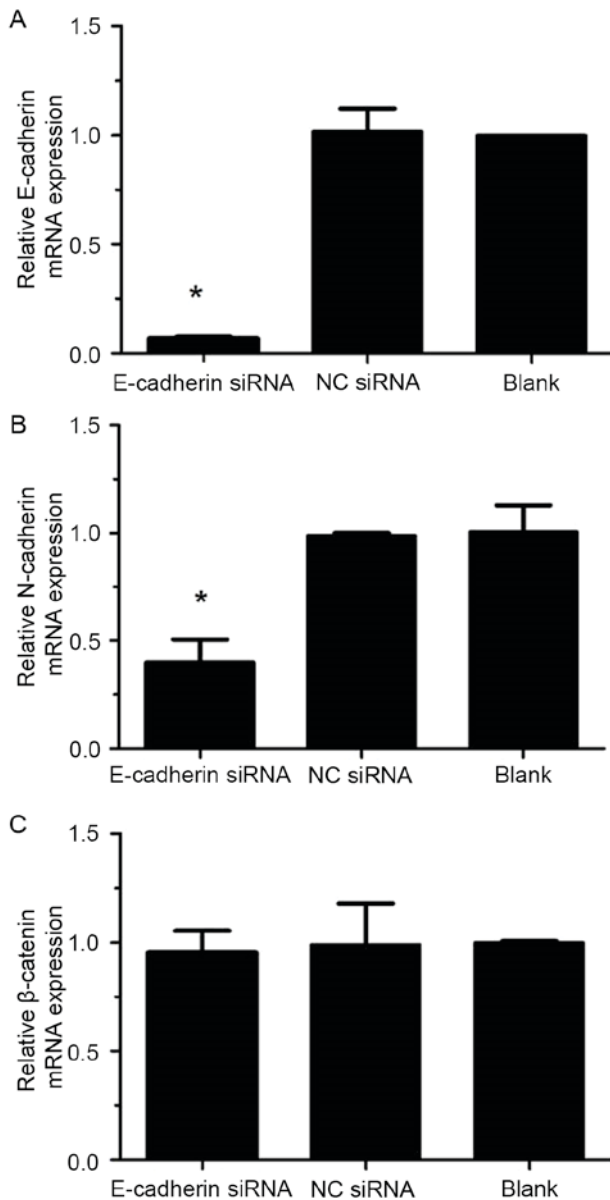


Figure 1. Reverse transcription-quantitative polymerase chain reaction analysis for E-cadherin, N-cadherin and β -catenin expression. The expression levels of (A) E-cadherin, (B) N-cadherin and (C) β -catenin in SRA01/04 human lens epithelial cells transfected with E-cadherin siRNA, NC siRNA or neither (Blank). Results are presented as the mean \pm standard error of the mean from three independent experiments; * P <0.05 vs. NC siRNA group. E, epithelial; N, neural; NC, negative control; siRNA, short interfering RNA.

membrane was probed overnight at 4°C with the following primary antibodies: rabbit anti-GAPDH (1:5,000; BM1623; Wuhan Boster Biological Technology, Ltd., Wuhan, China), rabbit anti-E-cadherin (1:500; 20874-1-AP; Wuhan Sanying Biotechnology, Wuhan, China), rabbit anti-N-cadherin (1:100; BA0673) and rabbit anti- β -catenin (1:100; BM3905) (both from Wuhan Boster Biological Technology, Ltd.). Following 3 washes with TBS + 0.05% Tween-20 (TBST), the membranes were incubated with the secondary horseradish peroxidase-conjugated goat-anti-rabbit immunoglobulin G antibody (1:5,000; 111-035-045; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h. Membranes were washed with 1X TBST buffer, and the formed immunocomplexes

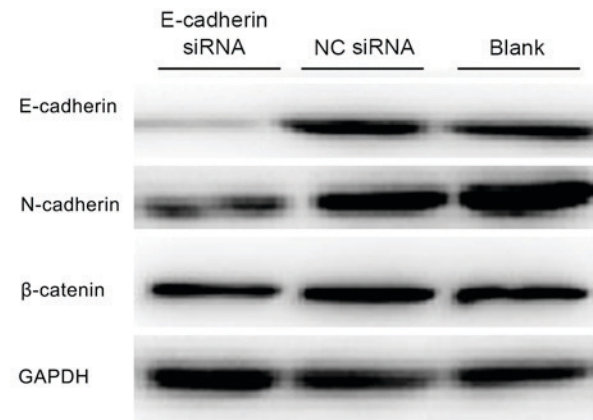


Figure 2. Western blot analysis for E-cadherin, N-cadherin and β -catenin protein expression. SRA01/04 human lens epithelial cells were transfected with E-cadherin siRNA, NC siRNA or neither (blank), and the protein expression of E-cadherin, N-cadherin and β -catenin were analyzed by western blotting. E, epithelial; N, neural; NC, negative control; siRNA, short interfering RNA.

were visualized by Enhanced Chemiluminescence reagent (Merck KGaA). Densitometric analysis was performed with Quantity One software (version 4.5.2) on the ChemiDoc XRS Gel Imaging system (both from Bio-Rad Laboratories, Inc., Hercules, CA, USA). Western blot analysis was repeated three times.

Statistical analysis. Statistical analysis was conducted using SPSS software version 11.0 (SPSS, Chicago, IL, USA). The data are presented as the mean \pm standard error of the mean. One-way analysis of variance (19) and the post-hoc Duncan's multiple range tests (20) were used to determine statistical significance. P <0.05 was considered to indicate a statistically significant difference.

Results

Gene expression levels of E-cadherin, N-cadherin and β -catenin. The mRNA expression levels of E-cadherin, N-cadherin and β -catenin in the E-cadherin siRNA, NC siRNA and Blank groups were detected by RT-qPCR (Fig. 1). Following 48 h E-cadherin siRNA transfection, the expression level of E-cadherin was significantly downregulated in the E-cadherin siRNA group compared with those in the NC siRNA and Blank groups (P <0.05; Fig. 1A). The mRNA expression level of N-cadherin in the E-cadherin siRNA group was also significantly reduced (P <0.05; Fig. 1B). However, the expression level of β -catenin was unaltered compared with those in the NC siRNA and Blank groups (P >0.05; Fig. 1C).

Protein expression of E-cadherin, N-cadherin and β -catenin. To investigate the protein levels of the E-cadherin, N-cadherin and β -catenin, western blotting was conducted (Fig. 2). Consistent with the RT-qPCR results aforementioned, the protein expression levels of E-cadherin and N-cadherin were reduced in the E-cadherin siRNA group compared with the NC siRNA and Blank groups, and the protein expression level of β -catenin had no notable changes.

Discussion

Cadherins are a group of calcium-dependent cell adhesion molecules that function through homophilic interactions to maintain cell-cell adhesion (21). The classical cadherins, which include E- and N-cadherin, as well as placental- and retinal-cadherin) are single-pass transmembrane glycoproteins that have a large degree of structural similarity and regulate cell-cell contacts through their extracellular amino terminus (22). E-cadherin and N-cadherin have been revealed to be pivotal mediators of cell adhesion that maintain cell-cell contacts (23). Immunofluorescence and *in situ* hybridization analyses demonstrated that E-cadherin, N-cadherin and P-cadherin have distinct expression patterns in the lens during development (24,25). A previous study revealed that the cooperative expression of N-cadherin and E-cadherin serve an important role in normal cell sorting and subsequent separation of the invaginated lens vesicle from the surface ectoderm (23). In the present study, E-cadherin expression was inhibited by transfecting human LECs with E-cadherin siRNA. RT-qPCR and western blot analysis revealed that the expression of E-cadherin was significantly downregulated following transfection with E-cadherin siRNA, which indicated that E-cadherin was successfully silenced. In addition, the expression of N-cadherin was also significantly decreased in E-cadherin siRNA transfected LECs.

A previous study reported that the decreased expression of E-cadherin and the induction of N-cadherin expression was a mediator of EMT (26). TGF- β was demonstrated to enhance tumor progression by promoting EMT, and TGF- β -induced EMT are characterized by a loss of junctional E-cadherin localization, overexpression of N-cadherin, the acquisition of a fibroblastoid morphology and increased cellular motility (27). Furthermore, the expression of N-cadherin was reported to lead to a mesenchymal phenotype (28), and the overexpression of N-cadherin, which is influenced by growth factors, has been implicated in EMT (29). Therefore, the present study hypothesized that LECs may be able to shut off EMT by downregulating the expression of N-cadherin, which may lead to a decrease in the occurrence of cataracts.

In addition, RT-qPCR and western blot analysis demonstrated that the mRNA and protein expression levels of β -catenin were unaffected in LECs transfected with E-cadherin siRNA. Previous studies have reported that cadherins are able to link the extracellular environment to the actin cytoskeleton through interactions with intracellular β -catenin (21,30). Therefore, the present study hypothesized that following E-cadherin siRNA exposure, LECs would not be able to form the β -catenin/E-cadherin complex, thus decreasing the adhesion among cells and resulting in EMT. Previous studies have demonstrated that β -catenin functions in lens fiber differentiation and is associated with both Wnt signaling and adhesion-related mechanisms that mediate early fiber and lens epithelium differentiation (31,32). Activation of the β -catenin/T cell factor (TCF) pathway and inhibition of E-cadherin were reported to be induced by upregulated gene 11, which regulates hypoxia-induced EMT (33). The loss of E-cadherin may give rise to increased β -catenin/TCF signaling, which cooperates with autocrine TGF- β signaling to sustain an undifferentiated mesenchymal phenotype (34).

However, in the present study, the expression of E-cadherin siRNA had no influence on the expression of β -catenin, which may be caused an insufficient transfection time of E-cadherin siRNA.

In conclusion, the present study investigated the potential action mechanism of E-cadherin in the regulation of human LEC transdifferentiation, which may occur via the regulation of N-cadherin levels. Further study is necessary to investigate the association between β -catenin with E-cadherin.

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