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Original Article

E-cadherin maintains the activity of neural stem cells and inhibits the migration

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Abstract: Cadherins are important adhesion molecules that mediate adhesions and communications between cells. These molecules participate in the formation and maintenance of multicellular organisms including the stem cells. E-cadherin is one of the classic cadherins which is reported to be essential for the survival and self-renewal of embryonic stem cells. Moreover, it could induce cell proliferation inhibitory signaling to regulate cell proliferation. In our study, we over-expressed and silenced E-cadherin in NSCs by lentiviral ways. Transgenic cells were confirmed by both quantitative RT-PCR and western blot. Results of MTT assay showed that over-expression of E-cadherin could enhance the cell activity. Furthermore, we performed Transwell chamber assay to analyze its role in regulation of cell migration. The results showed that the migration percent of over-expression cells was lower than control. Our results indicated that E-caherin would maintain the stemness of NSCs and reduce cells migration.

Keywords: Cadherins, E-cadherin, N-cadherin, neural stem cell, cell activity, cell migration

Introduction

The ability of cells to adhere and communicate to each other is very important for the formation and maintenance of multicellular organisms. During these processes, cadherin-catenin protein complexes represent as key regulators. E (epithelial)-, N (neural)-, and VE (vascular- endothelial)-cadherins are classic cadherins mostly studied in human pluripotent stem cells [1, 2]. E-cadherin is a transmembrane protein that mediates Ca^{2+}-dependent cell-cell adhesion in all epithelial tissues [3]. In human embryonic stem cells (hESCs), E-cadherin is essential for their survival and self-renewal. Its expression decreases immediately in undifferentiated hESCs after induction of differentiation, and its expression is considered as one of the undifferentiated properties during the hESCs maintenance [4-6]. In carcinoma lines, activation of E-cadherin resulted in retardation of cell proliferation [7]. It was later addressed that E-cadherin would induce proliferation inhibitory signaling [8]. N-cadherin is reported that its expression reduction led to premature differentiation of neurons and their migration away from the stem cell niche [9]. More and more researches show that cadherins take important roles in stem cell biology and it's crucial to know their detailed functions [1, 10].

Since little is known about function of E-cadherin in neural stem cells (NSCs), in our study, we focused its role on this aspect. We over-expressed and silenced this gene separately in NSCs, and analyzed its role in regulation of NSCs by MTT assay and Transwell chamber assay.

Materials and methods

NSC culture

The 15th-day fetal mice were killed by rapid decapitation. Immediately removal of the brain and its surrounding membranes, the hippocampus of the brain was used for NSC culturing. Dissociated tissue was digested with 0.125% trypsin (Invitrogen) for 30 minutes at 37°C. The digestion was terminated by DMEM/F12 (Gibco) with 10% FBS (Sigma), and then washed by DMEM/F12 twice. Cells were grown in DMEM/F12 culture media with 15 mM HEPES buffer

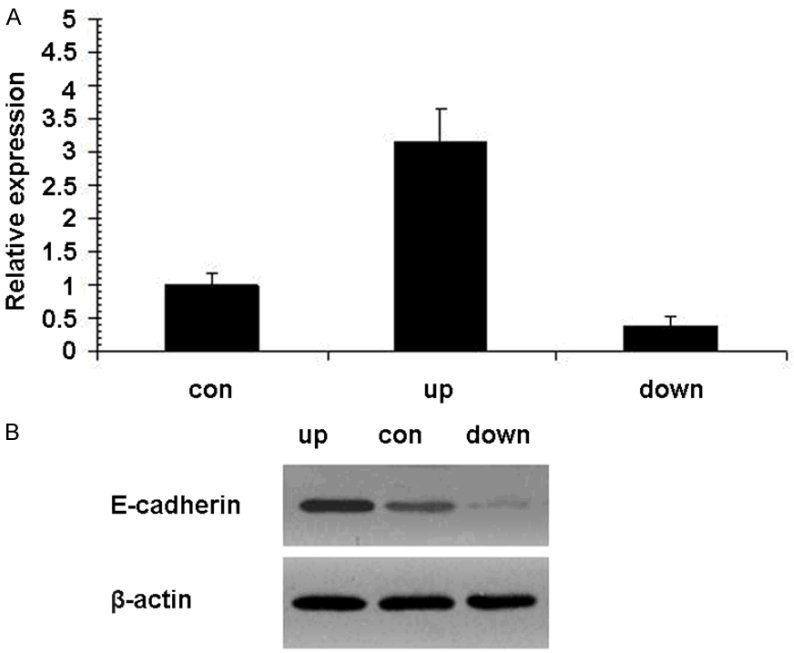

Figure 1. Over-expressed and RNAi of E-cadherin in NSCs. Confirmed by quantitative RT-PCR (A) and western blot (B). Con: control cells; up: over-expressed cells; down: RNAi cells.

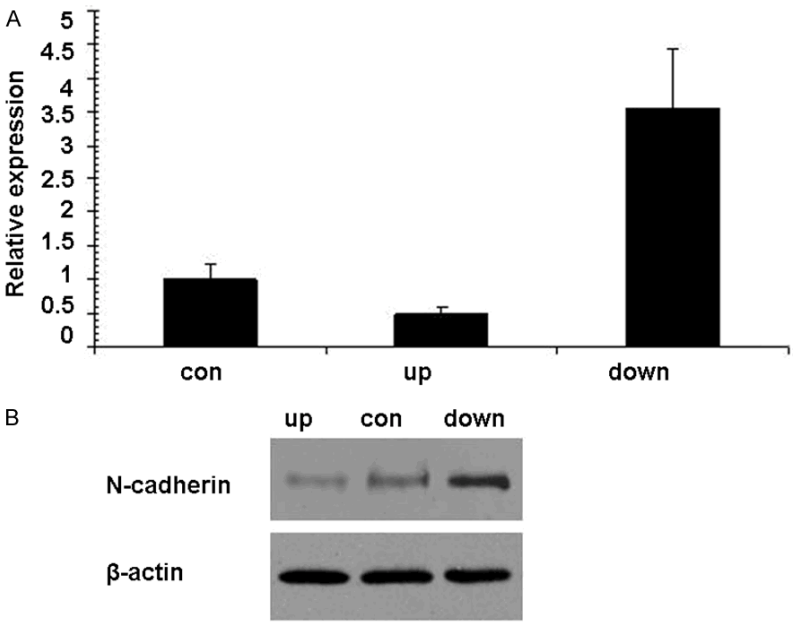

Figure 2. Expression level of N-cadherin in E-cadherin over-expressed and RNAi NSCs. N-cadherin expression level analyzed by quantitative RT-PCR (A) and western blot (B).

solution (Gibco), antibiotics supplemented with B27 supplement (1:50, Invitrogen) and 80 ng/ml FGF (PeproTech) at a clonal density of 4 × 10^5 cells/ml. The media was changed every 2 days.

Lentiviral constructs and the generation of active virus

Total RNA was extracted from tissues or cells using TRIzol Reagent (Invitrogen) and reverse transcribed into cDNA using the MMLV enzyme (Promega). Sequence of E-cadherin cDNA was obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). The full length of cDNA was amplified by primers as follows: F, 5'-AACGGATCCATGGGCCCTTGGAGCCGCAG-3'; R: 5'-GGGCTCGAGCTAGTCGTCCTCGCCGCCT-3'. RNAi was designed to target the coding sequence of E-cadherin. The sequences of RNAi were as follows: RNAi1, 5'-CCTGTCGAAGCAGGATTGCAA-3'; RNAi2, 5'-GACAACGTTTATTACTATGATGA-3'. These fragments were cloned into PHR-SIN-CSGW-H1a to generate expression constructs separately. After cotransfection of the expression clones and the Viral Power Packaging Mix (Life Technologies, Carlsbad, California, USA) into the 293-FT cell line (Life Technologies, Carlsbad, California, USA) to produce lentivirus, lentiviral supernatants were harvested. They were used to transducer NSC. Virus collection and propagation were performed according to the manufacturer's instructions (Life Technologies, Carlsbad, California, USA).

Quantitative real-time PCR

Real-time PCR was performed on an ABI 7300 real-time machine (Applied Biosystems). Cycle parameters were 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C 30 s, 72°C 30 s. The mRNA were detected by Quant qRT-PCR

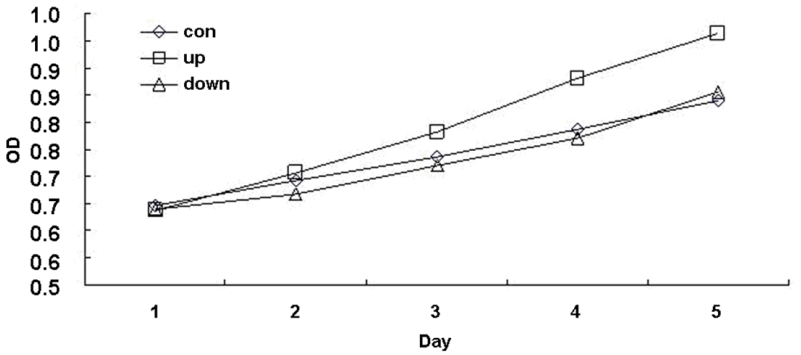

Figure 3. MTT assay in E-cadherin over-expressed and RNAi cells. It was performed in a following days. Absorbance values were measured at the wavelength of 570 nm.

(SYBR Green I) kit (Tiangen Biotech, Beijing, China) and normalized with the internal control β-actin. Primers were used as follows: E-cadherin F, 5'-GAGGTCTTTGAGGGATCTGTTG-3', R, 5'-GGCAGCATTGTAGGTGTTTATG-3'; N-cadherin F, 5'-GAGAGGAAGACCAGGACTATGA-3', R, 5'-TCTCGTCTAGCCGTCTGATT-3'; β-actin F, 5'-ACAGGATGCAGAAGGAGATTAC-3', R, 5'-ACAGTGAGGCCAGGATAGA-3'

The gene expression was calculated using $2^{-\Delta Ct}$ method. All data represent the average of three replicates.

Western blot analysis

Cells were collected on ice and washed by PBS. 1 × cell lysis buffer (Cell signaling Technology) was added to generate total cell extracts, and later collected the cell lysate by centrifugation. Samples were boiled by addition 6 × SDS sample buffer with 5% 2-ME for 10 min at 95°C. Equal amounts of proteins was separated electrophoretically by 10% SDS-PAGE and transferred onto PVDF membranes (Millpro). The membranes were incubated with the primary antibodies overnight at 4°C. Incubation with HRP-conjugated secondary antibodies for 2 h at room temperature, and then washed TBST and visualized by Odyssey infrared imaging system (Li-Cor Bioscience). The signals were analyzed by Image-Pro Plus 5.1.

MTT assay

NSCs were cultured in 96-well microplates at a density of 4 × 10^3 cells/well for 24 hours in a 37°C CO_2 incubator. MTT solution (5 mg/ml, Sigma) was added at a volume of 20 μl in each well and was incubated for 4 h. Discarded the solution and added 150 μl DMSO in each well. After blending for 10 min, absorbance values were measured at the wavelength of 570 nm. The MTT assay was performed for a following 8 days to draw the growth curve.

Transwell chamber assay

The cell migration was analyzed by Transwell chamber assay (Chemicon). NSCs were cultured on the upper chamber at a density of 1 × 10^5 cells/well in 200 μl serum free medium. 300 μl medium containing 10% serum was added to the lower chamber. After incubation for 24 h, the cells were fixed by NaCl-ethanol solution for 10 min and stained by 0.1% crystal violet solution (Sigma) for 10 min. Cells on the lower surface were counted with a light microscope.

Statistical analysis

The significance of differences between experimental groups was analyzed using the unpaired Student's *t*-test. *P*-values < 0.05 were considered significant.

Results

Expression level of N-cadherin in E-cadherin over-expressed and RNAi NSCs

To analyze the role of E-cadherin in NSCs, we constructed one over-expression vector and two RNAi vectors of this gene. Then we transferred them separately into NSCs by lentiviral ways. The quantitative RT-PCT showed that it was up-regulated in over-expressed cells and down-regulated in RNAi cells (**Figure 1A**). Furthermore, we performed western blot to confirm the results (**Figure 1B**). Thus, we obtained both over-expression and RNAi transgenic NSCs to do further study.

Since the N-cadherin is reported to be important in neural cells, we analyzed its expression level in our transgenic NSCs. Both quantitative RT-PCR and western blot showed that it was down-regulated in the over-expressed cells and up-regulated in the RNAi cells (**Figure 2**). These

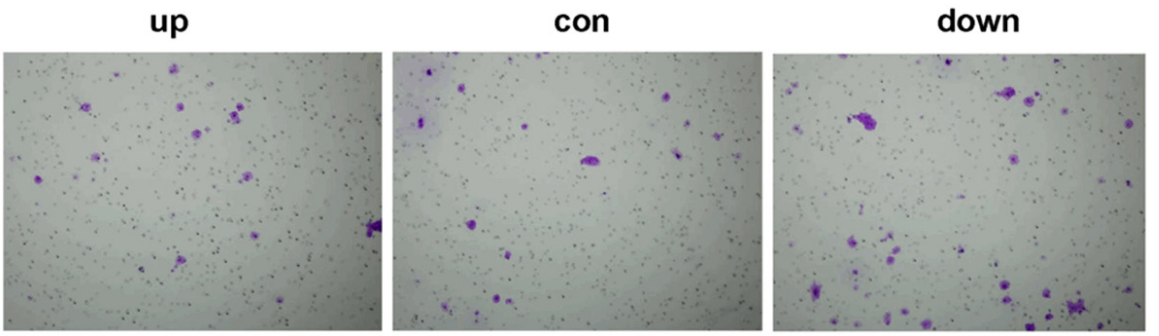

Figure 4. Transwell chamber assay in E-cadherin over-expressed and RNAi cells.

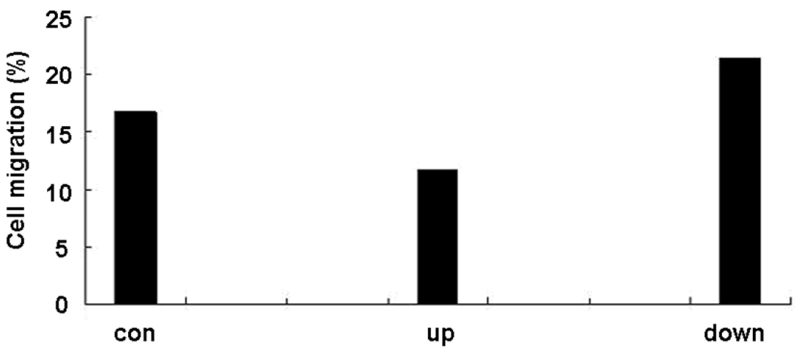

Figure 5. Analysis of cell migration percentage in E-cadherin over-expressed and RNAi cells. Cell migration was analyzed by Transwell assay.

results suggested that the expression pattern of N-cadherin was not similar like E-cadherin in NSCs.

E-cadherin enhanced the activity of NSCs

Later, we performed MTT assay to know whether E-cadherin would regulate the activity of NSCs. The cells were incubated with MTT solution for 4 h and measured the absorbance values at 570 nm. The MTT assay was performed in a following days. The results showed that there were no obvious differences between control cells and RNAi cells, but the vales measured in over-expression cells were higher than both control and RNAi cells (**Figure 3**). The results indicated that E-cadherin took a role in regulation of the activity of NSCs.

E-cadherin inhibited the migration of NSCs

Since the E-cadherin would regulate the activity of NSCs, we performed Transwell assay to know whether it could regulate the migration of NSCs. The results of Transwell assay showed that the migration percentage of NSCs was lower in over-expressed cells comparing to control, and it was higher in RNAi cells (**Figures 4** and **5**). It suggested that E-cadherin would inhibit the migration of NSCs.

Discussion

E-cadherin is one of the classic cadherins which takes crucial role in cell-cell adhesion [11]. It is known to be a tumor suppressor and could inhibit cell growth by inhibition of Wnt signaling and β-catenin/TCF transcriptional activity [12, 13]. In embryonic stem cells, E-cadherin levels are reduced when cells begin transitioning away from pluripotency and its expression is considered as one of the undifferentiated properties during the hESCs maintenance [2, 14, 15].

Since its role in neural stem cells is little known, we over-expressed and silenced it in NSCs to further study (**Figure 1**). Moreover, we analyzed another cadherin in the transgenic NSCs. The results showed that the expression of N-cadherin was lower than control in the over-expressed cells and higher in the RNAi cells (**Figure 2**). Later, we tested the cell activity in the transgenic cells by MTT assay. Comparing to control cells, over-expressed NSCs showed higher activity (**Figure 3**). The results indicated that E-cadherin could maintain the NSCs' activity, that is to say, it could maintain the stemness of NSCs. Furthermore, we performed Transwell assay to test its effect on cell migration. The migration percentage of over-expre-

ssed NSCs was lower than control, while the RNAi cells showed higher migration percentage (**Figures 4** and **5**). It suggested that E-cadherin would inhibit NSCs migration.

Combined with the results all above, our results indicated that E-cadherin would maintain the stemness of NSCs by enhancing cell activity and inhibiting cell migration.

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Disclosure of conflict of interest

None.

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