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GENERAL GYNECOLOGY

Knockdown of E-cadherin expression of endometrial epithelial cells may activate Wnt/β-catenin pathway in vitro

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Abstract
Purpose E-cadherin, a transmembrane glycoprotein mediating Ca^{2+}-independent homotypic cell–cell adhesion in epithelial cell, plays an essential role in metastasis. It has been postulated that E-cadherin downregulation is a crucial mechanism in the pathogenesis of endometriosis. To evaluate the effect on the cell behavior after knockdown of E-cadherin gene (CDH1) in cultured human endometrial epithelial cells (EECs) isolated from normal endometrium.
Methods EECs were isolated from the endometrial tissues of fertile woman who underwent total hysterectomy due to cervical intraepithelial neoplasia III. *CDH1* expression was knocked down by small hairpin RNA. The EECs transfected with empty vector served as control. Transwell assay was used to test EECs migration or invasion. qRT-PCR and western blot were used to detect mRNA and protein levels.
Results The results showed that knockdown of E-cadherin expression can increase cell migration and invasion, and upregulate mRNA and protein levels of β-catenin, cyclinD1, and c-myc.
Conclusions Down-regulation of E-cadherin expression may activate the Wnt/β-catenin pathway in endometrial cells, which may together participate in the occurrence of endometriosis.

Keywords Endometriosis · E-cadherin · Wnt/β-catenin pathway · Cell migration and invasion

Introduction

Endometriosis, defined as the presence of endometrial gland tissue and stroma outside the uterus, is a common disease with major symptoms including abdominal pain, dysmenorrhea, dyspareunia, and infertility, which seriously does harm to the psychological and physical health of women, especially the women in reproductive age. The pathogenesis of endometriosis remains largely unclear. However, it is believed that endometrial fragments circulate throughout fallopian tubes and enter the peritoneal cavity by retrograde menstruation [1]. These endometrial fragments are found at ectopic sties. The processes of adhesion, aggression, and angiogenesis can cause ectopic lesions. Hence, the migration and invasion of endometriotic cells may be related to the pathogenesis of endometriosis.

E-cadherin, a transmembrane glycoprotein, is expressed in all epithelial cells and is involved in mediating homotypic cell to cell adhesions, as well as maintaining epithelial cell polarity and integrity [2]. A loss of E-cadherin expression is a hallmark of the epithelial–mesenchymal transition (EMT) [3], which plays a crucial role in the early steps of metastasis. During this process, cells lose cell-to-cell contact through E-cadherin ablation, and thus, acquire increased mobility to spread into surrounding or distant tissues [4]. Thus, E-cadherin downregulation is closely related to the metastasis of epithelial cells. In endometriosis, previous studies suggest that E-cadherin-negative endometriotic cells display an invasive phenotype in a collagen invasion assay and similar metastatic ability to carcinoma cells [5,

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6]. Therefore, it has been postulated that E-cadherin down-regulation is a crucial mechanism in the pathogenesis of endometriosis.

The E-cadherin and cadherin–catenin complex of cytoplasm may modulate various signaling pathways, including Wnt/β-catenin signaling, which plays a significant role in embryonic development, tissue self-renewal, and various diseases [7, 8]. Many Wnt-responsive genes have a pivotal impact on cell proliferation, migration, and invasion [9]. These processes are also common in endometriosis [10]. A range of studies have shown that aberrant activation of the Wnt/β-catenin pathway may be involved in the pathophysiology of endometriosis [11–13]. However, the degree to which the Wnt/β-catenin pathway is activated by the loss of E-cadherin protein, and the degree to which the activated Wnt/β-catenin pathway plays a role in the development of endometriosis, remain unclear.

We hypothesize that the knockdown of E-cadherin expression results in the activation of the Wnt/β-catenin pathway in normal endometrial epithelial cells (EECs). To examine the impact on the Wnt/β-catenin pathway and the migratory and invasive abilities of EECs, a knockdown of E-cadherin expression by RNA interference in cultured EECs was conducted. This study may provide new clues for, not only understanding the pathogenesis of endometriosis, but also developing new therapies related to it.

Materials and methods

Patient recruitment and characterization

AII 18 patients with regular menstrual cycle, aged from 35 to 46 (the average age was 40 ± 4 years) recruited in this IRB approved study underwent a total hysterectomy. This is a common clinical procedure in response to a cervical intraepithelial neoplasia III. More specifically, all obtained samples were confirmed by a postoperative pathological diagnosis, the results of which suggest that two samples were in a proliferative phase and 16 a secretory phase. None of the subjects had received any hormone therapy prior to surgery. Finally, this study was approved by Fourth Hospital's Ethics Committee, Hebei Medical University, and informed consent was obtained from all recruited subjects.

Primary culture of endometrial epithelial cells

The endometrial biopsies were placed into a Dulbecco modified Eagle medium/Ham F-12(DMEM F/12) media (HyClone, Utah, USA) and digested in a mixed digestion suspension containing type I collagenase (1 g/L; Sigma, MO, USA) and Trypsin (0.25%; Thermo, Waltham, USA). After filtering the digested suspension, the tissues and cells on the filter were rinsed and eluted. The precipitant of the eluted suspension was found to be mostly epithelial cells. After Trypan Blue staining was performed on the sampled culture medium, cell density was calculated and adjusted to 400 cells/cm^3. Finally, these cells were cultured with 20% fetal bovine serum (FBS) in an incubator with 5% CO_2 and saturated humidity at 37 °C.

Immunocytochemistry

Immunohistochemistry was performed on the cultured cells when 70% confluence was reached. Nonspecific binding was blocked with 10% Bovine Serum Albumin (BSA) at 37 °C for 30 min. These cells were then incubated overnight at 4 °C using rabbit anti-CK 19 monoclonal antibodies (1:100; Abcam, CA, USA), extensively washed in 0.1% Tween 20, and then incubated with an anti-rabbit biotinylated secondary antibody (1:400; KPL, MD, USA) at room temperature for 60 min. Finally, these cells were coated with glycerol and imaged with a fluorescence microscope.

Knockdown of CDH1 in EECs by a small hairpin RNA (shRNA)

The RadE5-CDH1-shRNA vector and empty vector were purchased from GeneSil (Wuhan, China). The two siRNA oligonucleotide sequences for the E-cadherin gene (CDH1) were 5′-GAACGAGGCTAACGTCGTAAT-3′ and 5′-ATA CCAGAACCTCGAACTATA-3′. The negative control sequences were 5′-AGTCGTAGTAGCGATGTGG-3′ and 5′-CAACAAGATGAAGAGCACC-3′. The cells were plated with 70–80% confluency and transfected with 5.0×10^9 pfu adenoviral vectors according to manufacturer instructions. The efficiency of the CDH1 knockdown was confirmed by the qRT-PCR procedure. Finally, the transfected cells were used for various assays 48 h after transfection to allow for the effective knockdown of CDH1 in each experiment.

Transwell migration and invasion assay

Cell migration assays were performed using transwell chambers (Corning, NY, USA). The cell invasiveness of these assays was investigated using Falcon cell culture inserts coated with a Matrigel matrix (BD Bioscience, CA, USA). Approximately 4×10^4 cells in the FBS-free DMEM were loaded into the upper chambers. The lower chambers were filled with DMEM with 20% FBS concentration. The plates were incubated at 37 °C for 24 h. After this time, the non-invading cells in the top chamber were removed with swabs. The cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. Finally, images were captured using an Olympus DX41

inverted microscope, with cells in five visual fields per insert counted and photographed (400× magnification).

RNA extraction and quantitative real-time PCR (qRT-PCR)

All the RNA was extracted using Trizol (Thermo). 5ug of this was reversely transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit according to manufacturer protocols (Thermo). The qRT-PCR procedure was performed using an AceQTM qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) in Stratagene Mx3005P real-time PCR machine. The primer sequences are shown in Supplementary Table 1. The qRT-PCR program was set to an initial denaturation at 95 °C for 5 min, 40 reaction cycles, with each cycle consisting of a denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and then elongation at 72 °C for 30 s. β-actin mRNA served as the internal control. The mRNA levels of target genes were quantified using the following equation: $\Delta CT = CT(gene) - CT(\beta\text{-actin})$.

Western blot

Cells were lysed in a RIPA buffer solution (Bestbio, Shanghai, China) supplemented with protease inhibitors. Protein samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. These membranes were blocked in 5% non-fat milk for 1 h at room temperature and incubated in a corresponding primary antibody (Supplementary Table 2) at 4 °C overnight, followed by an anti-rabbit secondary antibody for 2 h at room temperature in a darkroom. Western blot signals were detected using an Odyssey infrared laser imaging system (Li-COR, Nebraska, USA) and exposed to chemiluminescent film. The relative protein levels were calculated by dividing the grayscale value of the target protein band by that of the GAPDH band.

Statistical analysis

Statistical analysis was performed using the software SPSS 22.0 (SPSS, Chicago, IL, USA). The data was shown in a mean ± SEM. The continuous variables were analyzed using a *t* test, with $P < 0.05$ (two-sided) used as the criterion for statistical significance.

Results

CDH1 knockdown effect on E-cadherin expression in EECs

The positive signals of the dense green fluorescent protein (GFP) indicated the high efficiency (> 70%) of adenoviral vector transfection in cultured human EECs (Fig. 1a) at 48 h after transfection (Fig. 1b). The qRT-PCR result showed that the level of CDH1 mRNA present in the EECs-RNAi group was significantly smaller than that present in the EECs-control group ($P = 0.003$) (Fig. 1c). This result was further validated in protein expression ($P = 0.011$) (Fig. 1d, e).

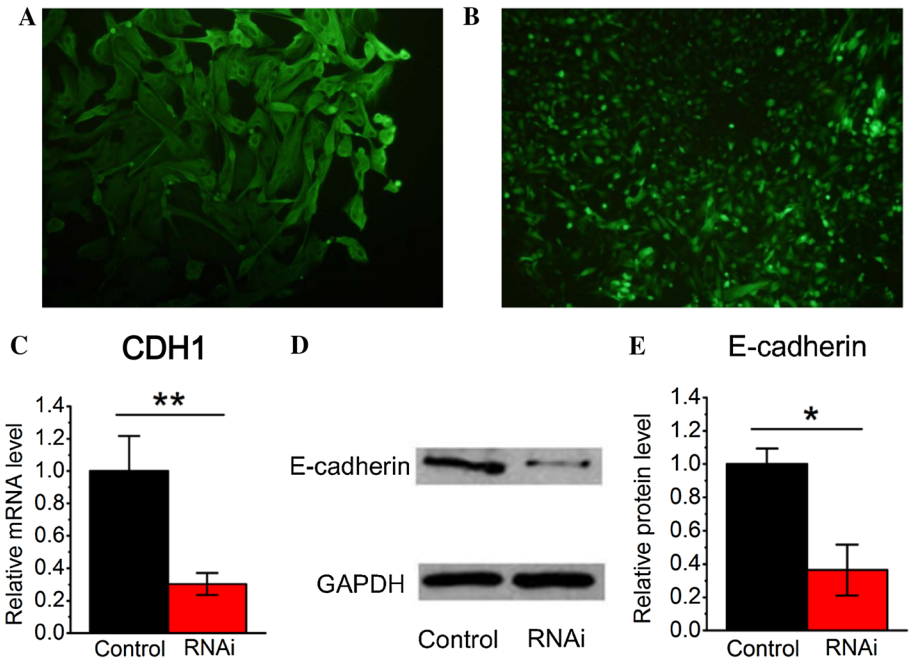

Fig. 1 Effect of CDH1 knockdown on E-cadherin expression in EECs. **a** The anti-CK19 signals in isolated human EECs. **b** The GFP signals (> 70%) in cultured human EECs after being transfected with adenoviral vector. **c** The E-cadherin gene mRNA levels in EECs-RNAi and EECs-control groups ($P = 0.003$). **d** Representative western blot results showing E-cadherin levels in EECs-RNAi and EECs-control groups. **e** E-cadherin protein level was significantly decreased in EECs-RNAi group ($P = 0.011$). $^*P < 0.05$, $^{**}P < 0.01$

CDH1 knockdown effect on the invasion and migration ability of EECs

The knockdown of CDH1 may significantly increase the invasion and migration ability of EECs. The transwell assay showed that the invasion and migration ability of the EECs present in the EECs-RNAi group were significantly higher compared with those of the EECs control group ($P = 0.005$ and $P = 0.009$) (Figs. 2, 3).

CDH1 knockdown effect on β-catenin expression

Compared with the EECs-control group, the knockdown of CDH1 in the EECs-RNAi group led to a 59.4% ($P = 0.006$) increase of β-catenin at an mRNA level (Fig. 3a), and a 84.7% increase ($P = 0.007$) at a protein level (Fig. 3b, c).

CDH1 knockdown effect on target genes of the Wnt/β-catenin pathway

We examined whether the expression of target genes in the Wnt/β-catenin pathway was also affected in the human EECs after the knockdown of CDH1. Cyclin D1 and c-myc are the two important downstream genes in the Wnt/β-catenin pathway. The qRT-PCR results showed that the mRNA levels of cyclin D1 and c-myc were increased by 72.0% ($P < 0.001$) (Fig. 4a) and 93.4% ($P = 0.034$) (Fig. 4d) in the EECs-RNAi group, respectively, compared with those in the EECs-control group. The western blot results showed that the protein levels in the EECs-RNAi group of Cyclin D1 was increased by 33.8% ($P = 0.002$) (Fig. 4b, c), and c-myc 44.9% ($P = 0.004$) (Fig. 4e, f).

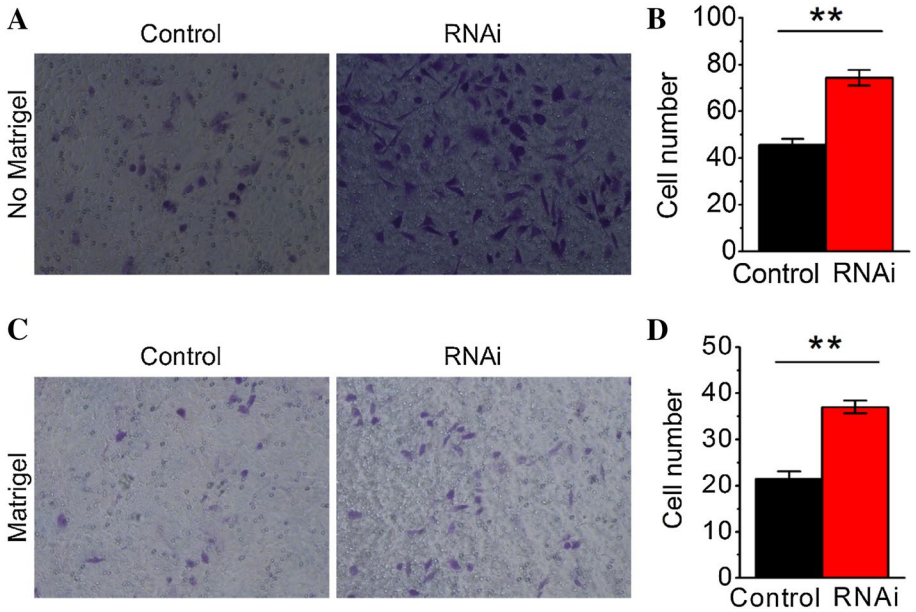

Fig. 2 Effect of CDH1 knockdown on invasion and migration ability of EECs. Representative crystal violet staining results of human EECs in the transwell chamber without (**a**) or with (**c**) matrigel cover. The number of EECs penetrating polycarbonate membrane was significantly increased in EECs-RNAi group in the transwell chamber without ($P = 0.005$) (**b**) or with (**d**) ($P = 0.009$) matrigel cover. $^{**}P < 0.01$

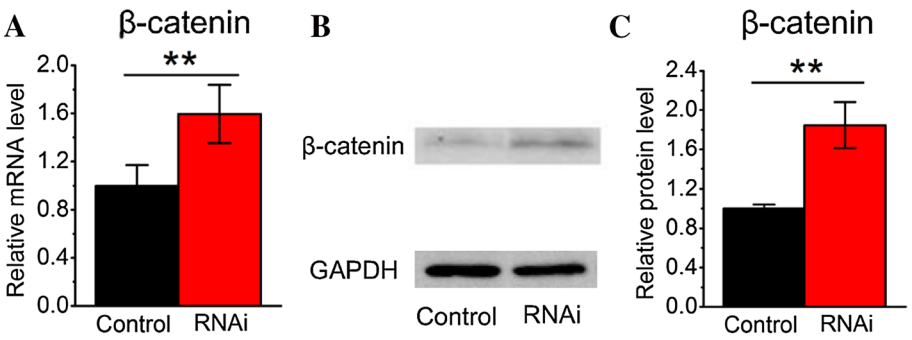

Fig. 3 Effect of CDH1 knockdown on β-catenin expression. **a** Compared with EECs-control group, knocking down E-cadherin expression in EECs-RNAi group led to the increase of β-catenin mRNA level (increased by 59.4%, $P = 0.006$). **b** Representative western blot results showing β-catenin levels in EECs-RNAi and EECs-control groups. **c** The β-catenin protein level was significantly increased in EECs-RNAi group (by 84.7%, $P = 0.007$). $^{**}P < 0.01$

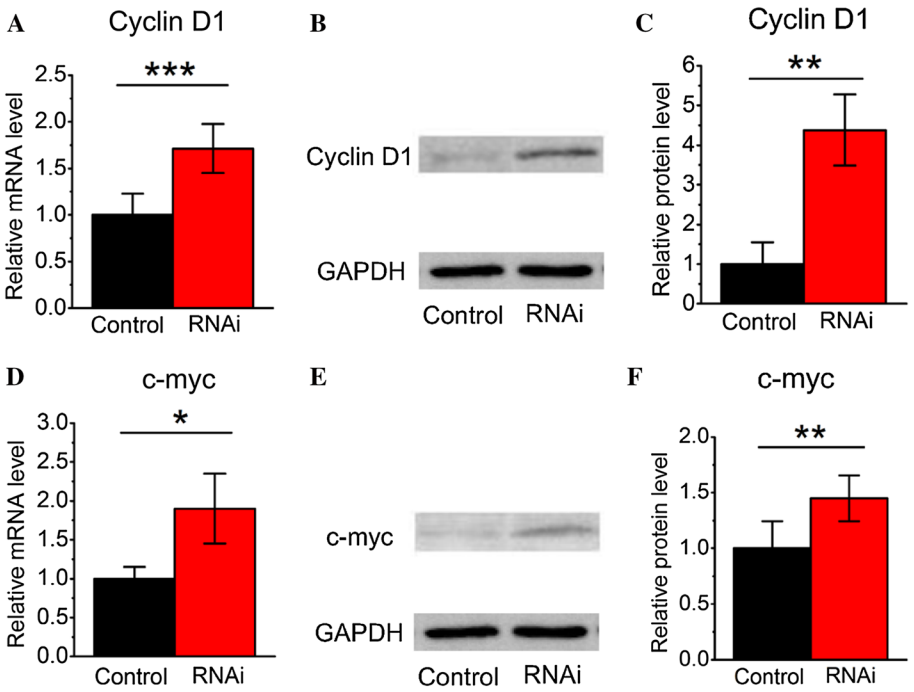

Fig. 4 Effect of CDH1 knockdown on target genes of the Wnt/β-catenin pathway. Compared with EECs-control group, knocking down E-cadherin expression in EECs-RNAi group led to the increase of cyclin D1 ($P < 0.001$) (**a**) and c-myc ($P = 0.034$) (**d**) mRNA levels. Representative western blot results showing cyclin D1 (**b**) and c-myc (**e**) levels in EECs-RNAi and EECs-control groups. The cyclin D1 ($P = 0.002$) (**c**) and c-myc ($P = 0.004$) (**f**) levels were significantly increased in EECs-RNAi group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Discussion

This study presents two significant findings. Firstly, the knockdown of the E-cadherin expression in the cultured human EECs can lead to an increase in cell migration and invasion. This knockdown can lead to an increase in up-regulated mRNA, specifically the protein levels of β-catenin, cyclin D1, and c-myc. Secondly, these results indicate that nonmalignant endometrial cells may acquire migratory and invasive abilities after a loss of E-cadherin expression, and the down-regulation of the E-cadherin expression may activate the Wnt/β-catenin pathway by releasing β-catenin in the EECs. It is believed that this study was first to investigate the effect E-cadherin has on the knockdown behavior of human EECs in vitro.

Contradictory results have been reported by some studies in relation to an E-cadherin expression associated with endometriosis. In relation to the endometrium, some studies report a reduction of an E-cadherin expression that is associated with endometriosis [14, 15], while others report no differences in an E-cadherin expression [16, 17]. However, in vitro studies have shown that primary negative E-cadherin expression epithelial cells extracted from peritoneal endometriosis biopsies are invasive, whereas noninvasive endometrial epithelial cells were E-cadherin positive. This E-cadherin negative epithelial cell type was increased in sections of endometriosis biopsies as compared with sections of a normal endometrium [5]. Therefore, it was proposed that the lack of E-cadherin expression is crucial for the invasiveness of endometriotic cells both in vitro and in vivo. In this study, we used an RNA interference to specifically down-regulate an E-cadherin protein expression extracted from cultured human EECs found in a normal endometrium. The results demonstrate that the invasion and migration ability of EECs had significantly increased. This suggests that normal endometrial cells may acquire migratory and invasive abilities after a loss of E-cadherin expression.

In addition, we found that the knockdown of an E-cadherin expression can result in an increase of up-regulated β-catenin, cyclinD1, and c-myc genes, as well as an increase of mRNA and protein expression in the EECs. β-catenin is a critical component of cell to cell adhesions, on which it forms a dynamic link between E-cadherin and the actin cytoskeleton of cytoplasm [18, 19]. The reduced expression of E-cadherin leads to both the decomposition of the E-cadherin–catenin complex and an increase in free cytoplasmic β-catenin. This may migrate into the nucleus and trigger the expression of EMT-inducing transcription factors, and increase cell migration and invasiveness [20, 21]. β-catenin is also a critical component of the Wnt/β-catenin pathway because it interacts with T-cell factor-1/lymphoid enhancing factor-1 (Tcf/LEF) transcription factors, leading to the transcriptional activation of Wnt-responsive genes, including both cyclinD1 and c-myc, which regulate cell proliferation and differentiation [22, 23]. It has been reported that aberrant activation of Wnt/β-catenin pathway may contribute to endometriosis [24]. Another study [25] had demonstrated that the endometriosis patients treated with PKF 115-584, a small-molecule antagonist of the Tcf/β-catenin complex, had higher inhibitory effects in menstrual endometrial

epithelial and stromal cells on migration and invasion, than the patients without endometriosis. This was the further evidence that aberrant activation of Wnt/β-catenin pathway may contribute to endometriosis by increasing the migration and invasion of menstrual endometrial cells. Based on the previous studies, we speculate that down-regulation of E-cadherin expression may activate Wnt/β-catenin pathway in endometrial tissue, which was involved in migration, invasion, and ectopic implantation of endometrial cells.

This study may have several limitations. Firstly, the culture of EECs is quite demanding for restricted tissue collection, and for advanced culturing technology to extract. This may bring about inadequate numbers and a relative low purity (approximately 80%) of the EECs. Secondly, the design of the experiment can be improved. Given the shortage of EECs made available, this study failed to prove an accumulation of β-catenin in a cell nucleus, and that an E-cadherin down-regulation improves EECs proliferation.

In conclusion, this study reveals that a down-regulation of E-cadherin expression results in increased cell migration and invasion, the up-regulation of a β-catenin expression, and an increase in two critical downstream genes in the Wnt/β-catenin pathway: cyclin D1 and c-myc as found in human ECCs. These results may provide new clues for understanding the pathogenesis of human endometriosis, which may facilitate the development of new therapies for treating human endometriosis in the future.

Author contributions SK: Protocol development. YL: Protocol development and manuscript writing. XZ: Performed the experiments and manuscript writing. RZ: Data analysis. NW: Data analysis.

Compliance with ethical standards

Ethics approval This study was approved by Fourth Hospital's Ethics Committee, Hebei Medical University.

Informed consent Informed consent was obtained from all recruited subjects.

Conflict of interest The authors declare that there is no conflict of interest.

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