Knockdown of lncRNA TUG1 protects lens epithelial cells from oxidative stress-induced injury by regulating miR-196a-5p expression in age-related cataracts

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Abstract. Oxidative stress plays an important role in the pathogenesis of cataracts. Under oxidative stress, apoptosis of lens epithelial cells (LECs) is activated, which may cause lens opacity and accelerate the development of cataracts. Long non-coding RNA (lncRNA) and microRNA (miRNA/miR) are involved in cataracts. Previous studies have demonstrated that lncRNA taurine upregulated 1 (TUG1) promotes cell apoptosis induced by ultraviolet radiation by downregulating the expression of miR-421. However, the mechanism underlying TUG1 in age-related cataract remains to be elucidated. The present study aimed to investigate the effect of TUG1 in age-related cataracts and to determine the related underlying molecular mechanism. In the present study, the association between TUG1 and microRNA (miR)-196a-5p was predicted using StarBase and verified using a dual luciferase reporter assay in 293 cells. The LEC line SRA01/04 was exposed to 200 µM hydrogen peroxide (H₂O₂) for 24 h to establish an in vitro oxidative stress model. The mRNA expression levels of TUG1 and miR-196a-5p were analyzed using reverse transcription-quantitative PCR, whilst cell viability and apoptosis were determined using MTT and flow cytometry assays, respectively. The protein expression levels of cleaved caspase-3 and caspase-3 in SRA01/04 cells were determined using western blotting. The results of the present study revealed that TUG1 directly targeted miR-196a-5p expression. In addition, the expression levels of miR-196a-5p were downregulated in SRA01/04 cells following oxidative stress, whilst TUG1 expression was upregulated. Cell transfection with TUG1-small interfering RNA (siRNA) upregulated miR-196a-5p expression levels in SRA01/04 cells, which was reversed following co-transfection with the miR-196a-5p inhibitor. Transfection with TUG1-siRNA also reduced the levels of H₂O₂-induced oxidative damage in SRA01/04 cells, which was demonstrated by increased cell viability, reduced levels of apoptosis and downregulated cleaved caspase-3 levels. Conversely, transfection with the miR-196a-5p inhibitor reversed these effects aforementioned. Overexpression of miR-196a-5p reduced H₂O₂-induced oxidative damage in SRA01/04 cells. In conclusion, findings from the present study suggested that knocking down TUG1 expression may protect LECs from oxidative stress-induced apoptosis by upregulating the expression of miR-196a-5p.

Introduction

Cataracts can occur due to various factors, including aging, a familial history, immune and metabolic abnormalities, trauma to the eye and exposure of the eye to poison or radiation (1,2). Cataracts can lead to lens protein degeneration and opacity (3). At present, age-related cataracts is the most common type of cataracts (4-6). Cataracts commonly develop in middle-aged and elderly individuals aged >50, where the incidence increases with age (7). Age-related cataracts are associated with the natural degeneration of the lens, which occurs over time during old age (8). Clinically, age-related cataracts can be divided into three subtypes, namely cortical, nuclear and posterior subcapsular cataracts (9,10). There is currently no strict distinction between these various subtypes of age-related cataracts, but all can progress into the total calcification of the lens (11). Cortical cataracts are the most common type of age-related cataracts, accounting for 65-70% of all age-related cataract cases, followed by nuclear cataracts, accounting for 25-35% of cases and finally subcapsular opacity cataracts, which are relatively rare and only account for 5% of all age-related cataracts (12,13). Age-related cataracts is one of the main causes of blindness, and the incidence rate increases with age. Worldwide, patients aged 43-54 years have an incidence rate of 8.3%, compared with an incidence as high as 70.5% in patients >75 years-old (14,15). It was previously discovered that oxidative stress serves an important role in the pathogenesis of cataracts. Under oxidative stress, apoptosis is induced in lens epithelial cells (LECs), which was found to promote the opacification of the lens and accelerate the development of cataracts (16,17). Therefore, the present study used human LECs to study the pathogenesis of age-related cataracts.
Long non-coding RNAs (lncRNAs) are a type of non-coding RNA that are >200 nucleotides in length but lack protein coding ability (18,19). Although lncRNAs do not generally encode protein, they participate in the regulation of protein-coding gene expression at multiple levels, including epigenetic, transcriptional and post-transcriptional regulation (20,21). MicroRNAs (miRNAs/miRs) are a type of endogenous non-coding small RNA that are 21-25 nucleotides in length and exist in both animals and plants (22,23). The tissue specificity of miRNA and the time at which they are expressed determines their functional specificity in tissues and cells (24). This suggests that miRNAs can serve important roles in the regulation of cell proliferation, in addition to having a key role in the regulation of post-transcriptional gene expression. It was previously reported that the dysregulated expression levels of lncRNAs and miRNAs were associated with the occurrence of cataracts (25-27). For example, Chen et al (25) demonstrated that increased expression of miR-26a and miR-26b inhibited lens fibrosis and cataract formation by regulating the Jagged-1/Notch signaling pathway. Zhang et al (26) previously found that downregulation of miRNA-133b suppressed apoptosis of LECs by upregulating BCL2L2 in age-related cataracts. In addition, the expression of TUG1 in the anterior lens capsules of age-related cataract were revealed to be significantly higher compared with normal anterior lens capsules, where TUG1 promoted ultraviolet radiation-induced apoptosis by downregulating the expression of miR-421 (27).

miR-196a-5p has been studied in various diseases, including cancer, pre-eclampsia and postmenopausal osteoporosis (28-30). miR-196a-5p serves a key role in the regulation of post-transcriptional gene expression. It was demonstrated that increased expression of miR-26a and miR-26b significantly higher compared with normal anterior lens capsules, where TUG1 promoted ultraviolet radiation-induced apoptosis by downregulating the expression of miR-421 (27).

To establish an oxidative stress model, SRA01/04 cells were cultured in six-well plates at 37°C for 24 h and then transfected with 200 pmol/l control small interfering RNA (siRNA; sense, 5'-UUC UCC GGU UCU ACG UUC CAG CTT-3'; antisense, 5'-ACG UGA CAC GGU UCU ACG CTT-3'; Shanghai GenePharma Co., Ltd.), 200 pmol/l TUG1-siRNA (sense, 5'-CCAUCUCACAGGCUC UCA ATT-3'; antisense, 5'-TTGUAGUUGUUGGUACAA GUU-5'; Shanghai GenePharma Co., Ltd.), 50 nM inhibitor control (5'-CAGUACUUUUGUGAUUAGAA-3'; Shanghai GenePharma Co., Ltd.), 50 nM miR-196a-5p inhibitor (5'-CCC AACAAUGAAGUUCGUAA-3'; Shanghai GenePharma Co., Ltd.), 100 nM mimic control (sense, 5'-UACUCGGAA CGUUCACGUTT-3'; antisense, 5'-ACGUGACCGUU CGGAGATT-3'; Shanghai GenePharma Co., Ltd.) or 100 nM miR-196a-5p mimic (sense, 5'-UAGGUUGUUCG UUUGUUGG-3'; antisense, 5'-CAACAAUGAAGUAC ACCAUUU-3'; Shanghai GenePharma Co., Ltd.) for 24 h using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection, the cells were collected to determine the transfection efficiencies by reverse transcription-quantitative PCR (RT-qPCR).

To determine the effect of TUG1-siRNA in SRA01/04 cells exposed to 200 µM H2O2, cells were divided into the following groups: i) Control group, with cells without any treatment; ii) H2O2 group, where the SRA01/04 cells were exposed to 200 µM H2O2 at 37°C for 24 h; iii) H2O2 + control-siRNA group, where SRA01/04 cells were transfected with control-siRNA for 24 h and then exposed to 200 µM H2O2 at 37°C for 24 h; iv) H2O2 + TUG1-siRNA group, where SRA01/04 cells were transfected with TUG1-siRNA for 24 h and then exposed to 200 µM H2O2 at 37°C for 24 h; v) H2O2 + TUG1-siRNA + inhibitor control group, where SRA01/04 cells were co-transfected with the TUG1-siRNA + inhibitor control for 24 h and then exposed to 200 µM H2O2 at 37°C for 24 h; and vi) H2O2 + TUG1-siRNA + miR-196a-5p inhibitor group, where SRA01/04 cells were co-transfected with TUG1-siRNA + miR-196a-5p inhibitor for 24 h and then exposed to 200 µM H2O2 at 37°C for 24 h.

To determine the effect of the miR-196a-5p mimic on SRA01/04 cells following exposure to 200 µM H2O2, cells were divided into the following groups: i) Control group, which consists of cells without any treatment; ii) H2O2 group, where SRA01/04 cells were exposed to 200 µM H2O2 at 37°C for 24 h; iii) H2O2 + mimic group, where SRA01/04 cells were transfected with the mimic control for 24 h and then exposed to 200 µM H2O2 at 37°C for 24 h; iv) H2O2 + mimic control group, where SRA01/04 cells were transfected with the mimic control for 24 h and then exposed to 200 µM H2O2 at 37°C for 24 h; v) H2O2 + miR-196a-5p mimic group, where SRA01/04 cells were transfected with miR-196a-5p mimic for 24 h and then exposed to 200 µM H2O2 at 37°C for 24 h.

miRNA target analysis and dual-luciferase reporter assay. The direct binding site between TUG1 and miR-196a-5p was identified using StarBase version 2.0 (http://starbase.sysu.edu.cn/). The 3'-untranslated region (UTR) sequences of TUG1 [TUG1-wild-type (WT), 5'-AUGCUAAUUU CAUCACUCU-3'], which included the target sequence for miR-196a-5p, or the mutated (MUT) target site (TUG1-MUT, 5'-AUGGUUGUUUACUUGAGAUU-3') were obtained by PCR using a Transcriptor First Strand cDNA Synthesis kit (cat. no. 0489686601; Roche Diagnostics GmbH). The thermocycling conditions were as follows: Incubation for 5 min at 25°C, followed by 60 min at 42°C. The 3'-UTR products were cloned into the pmiR Glo vector (Promega Corporation) to construct the TUG1-WT reporter vector. In addition, a TUG1-MUT reporter vector was also generated. 293 cells were obtained from the ATCC and cultured in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific,
Inc.) at 37°C with 5% CO2. Briefly, 293 cells were cultured for 24 h before being co-transfected with 1 ng TUG1-WT or 1 ng TUG1-MUT luciferase reporter gene plasmid and 100 nM miR-196a-5p mimic or 100 nM mimic control using Lipofectamine® 2000 reagent for 48 h. The relative luciferase activity was measured using a Dual Luciferase Reporter assay system (Promega Corporation), according to the manufacturer’s protocol. The results were normalized to Renilla luciferase activity.

RT-qPCR. Total RNA was extracted from cells using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific Inc.). Total RNA was reverse transcribed into cDNA using a Maxima First Strand cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). The reaction conditions for RT-PCR were as follows: 70°C for 5 min, 37°C for 5 min and 42°C for 60 min. qPCR was performed in an ABI Prism 7000 Real-Time PCR Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR™-Green qPCR Master mix (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The primers used for the qPCR were synthesized by Genescript and primer sequences were listed as the following: GAPDH forward, 5'-CTTGGTATCTGTTGAGGAGGCAGTAAGATGTCTT-3'; U6 forward, 5'-GCTCGGCAACACATATCTAAAAT-3' and reverse, 5'-CCCTTCAGGAATTTGCGTGTCAT-3'; TUG1 forward: 5'-GACCGTCAATGACCCTTCC-3' and reverse, 5'-TGCCGGTGAATGCTCTTGGTGTC-3'; miR-196a-5p forward, 5'-CCGAGGTAGGATTTGCTATGGT-3' and reverse, 5'-GTCCAGGTCCAGGATTTGCTATC-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation for 5 min at 95°C; followed by 40 cycles for 10 sec at 95°C and 30 sec at 60°C. GAPDH or U6 were used as the internal controls for TUG1 and miR-196a-5p, respectively. The relative mRNA expression levels of TUG1 and miR-196a-5p were calculated using the 2^ΔCq method (34).

MTT assay. MTT assay was performed to evaluate cell viability. Briefly, 24 h after cell transfection, SRA01/04 cells were exposed to 200 µM H2O2 at 37°C for another 24 h, before the cells were seeded into a 96-well plate (1x10^4 cells per well). They were then treated with 10 µl 5 mg/ml MTT solution (Beyotime Institute of Biotechnology) per well and incubated at 37°C for an additional 4 h. Following incubation, the medium was removed and 100 µl DMSO was added to each well to dissolve the formazan product. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Flow cytometry analysis of apoptosis. Flow cytometry was used to detect cell apoptosis. Briefly, following transfection, the cells were harvested by trypsinization and resuspended in 1X buffer (Annexin V-FITC/PI apoptosis detection kit; Beyotime Biotechnology). In total, 100 µl of this cell suspension (1x10^5 cells) was incubated with 5 µl Annexin V-FITC and propidium iodide at 4°C in the dark for 15 min. The stained cells were analyzed using a BD FACSCalibur™ flow cytometer (BD Biosciences) and FlowJo software (version 7.2.4; FlowJo LLC).

Western blotting. Total protein was extracted from SRA01/04 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the lysate was centrifuged at 4°C at 10,000 x g for 15 min to obtain the total protein. Total protein (40 µg per lane) was quantified using a BCA protein assay kit (Bio-Rad Laboratories, Inc.) and separated by 10% SDS-PAGE. The separated proteins were subsequently transferred onto PVDF membranes and blocked with 5% non-fat milk diluted in PBS-0.1% Tween-20 (PBST) solution at room temperature for 1 h. The membranes were then incubated with the following primary antibodies overnight at 4°C: Anti-cleaved caspase-3 (cat. no. ab32042; 1:1,000; Abcam), anti-caspase-3 (cat. no. ab32351; 1:1,000; Abcam) and anti-GAPDH (cat. no. ab9485; 1:1,000; Abcam). Following primary antibody incubation, the membranes were washed three times with PBST and incubated with a goat anti-rabbit IgG H&L (HRP) pre-adsorbed (cat. no. ab97080; 1:2,000; Abcam) for 1 h at room temperature. Protein bands were visualized using an ECL substrate (Cytiva), according to the manufacturer's protocol on an ImageQuant800 western blotting imaging system (Amersham; Cytiva).

Statistical analysis. Data are presented as the mean ± SD from three independent experiments. Statistical differences among groups were determined using an unpaired Student's t-test or one-way ANOVA followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results.

TUG1 is a direct target gene of miR-196a-5p. Analysis using the StarBase database identified a binding site between TUG1 and miR-196a-5p (Fig. 1A), where this binding between TUG1 and miR-196a-5p was validated using a dual luciferase reporter assay (Fig. 1B). Compared with that in cells co-transfected with
the mimic control and TUG1-WT, the luciferase activity of cells co-transfected with miR-196a-5p mimic and TUG1-WT was significantly reduced (Fig. 1B). By contrast, the luciferase activity of cells co-transfected with miR-196a-5p mimic and TUG1-MUT demonstrated no significant changes compared with the luciferase activity of cells co-transfected with mimic control and TUG1-MUT (Fig. 1B).

Expression levels of TUG1 and miR-196a-5p in SRA01/04 cells after H₂O₂ treatment. The expression levels of TUG1 and miR-196a-5p in SRA01/04 cells were analyzed using RT-qPCR after cells were exposed to H₂O₂ for 24 h. As shown in Fig. 2A and B, compared with those in the control group, the expression levels of TUG1 were significantly upregulated in the H₂O₂ group, whilst the expression levels of miR-196a-5p were significantly downregulated.

TUG1 negatively regulates the expression of miR-196a-5p in SRA01/04 cells. SRA01/04 cells were transfected with TUG1-siRNA or the miR-196a-5p inhibitor for 24 h before RT-qPCR was performed to determine the transfection efficiency. Compared with those in the control-siRNA or inhibitor control groups, transfection with TUG1-siRNA or the miR-196a-5p inhibitor significantly downregulated the expression levels of TUG1 and miR-196a-5p in SRA01/04 cells, respectively (Fig. 3A and B). In addition, compared with those in the control-siRNA group, transfection with TUG1-siRNA significantly upregulated the expression levels of miR-196a-5p in SRA01/04 cells, which was significantly reversed following co-transfection with the miR-196a-5p inhibitor (Fig. 3C).

miR-196a-5p inhibitor reverses the effects of TUG1-siRNA on H₂O₂-induced oxidative damage of SRA01/04 cells. SRA01/04 cells were exposed to H₂O₂ following transfection for 24 h and were subsequently divided into the following six groups: i) Control group; ii) H₂O₂ group; iii) H₂O₂ + control-siRNA group; iv) H₂O₂ + TUG1-siRNA group; v) H₂O₂ + TUG1-siRNA + inhibitor control group; and vi) H₂O₂ + TUG1-siRNA +
miR-196a-5p inhibitor group. Compared with those in the control group, TUG1 expression levels were significantly upregulated in the H2O2 group, whilst the expression levels of miR-196a-5p were significantly downregulated (Fig. 4A and B). Compared with those in the H2O2 + control-siRNA group, TUG1 expression levels were significantly downregulated in the H2O2 + TUG1-siRNA group (Fig. 4A). In addition, compared with those in the H2O2 + control-siRNA group, miR-196a-5p expression levels were found to be significantly upregulated in the H2O2 + TUG1-siRNA group (Fig. 4B). Notably, this effect was reversed following co-transfection with the miR-196a-5p inhibitor (Fig. 4B).

In addition, the viability, apoptosis, and expression levels of cleaved caspase-3 and caspase-3 were analyzed in SRA01/04 cells. Compared with that in the control group, the viability of cells in the H2O2 + TUG1-siRNA group was significantly increased (Fig. 4C), whilst the cell apoptotic rate, protein expression levels of cleaved caspase-3 and the cleaved caspase-3/caspase-3 ratio were significantly reduced in the H2O2 group (Fig. 4D and E). By contrast, compared with that in the H2O2 + control-siRNA group, the viability of cells in the H2O2 + TUG1-siRNA group was significantly increased (Fig. 4C), whilst the cell apoptotic rate, protein expression levels of cleaved caspase-3 and the cleaved caspase-3/caspase-3 ratio were significantly reduced in the H2O2 + TUG1-siRNA + inhibitor control group (Fig. 4D and E). All of these effects aforementioned were found to be significantly reversed following co-transfection with the miR-196a-5p inhibitor.

miR-196a-5p attenuates H2O2-induced oxidative damage in SRA01/04 cells. SRA01/04 cells were exposed to H2O2 following 24 h of transfection and subsequently divided into the following four groups: i) Control group; ii) H2O2 group; iii) H2O2 + mimic control group; and iv) H2O2 + miR-196a-5p mimic group. Transfection with the miR-196a-5p mimic significantly upregulated the expression levels of miR-196a-5p in SRA01/04 cells (Fig. 5A), suggesting the successful transfection of the miR-196a-5p mimic. Subsequently, the underlying molecular mechanism of miR-196a-5p in SRA01/04 cells was investigated. Compared with those in the control group, miR-196a-5p
expression levels and cell viability were significantly reduced in the H$_2$O$_2$ group (Fig. 5B and C), whilst the apoptosis levels, protein expression levels of cleaved caspase-3 and the cleaved caspase-3/caspase-3 ratio were all significantly increased (Fig. 5D-G). Conversely, compared with those in the H$_2$O$_2$ + mimic control group, miR-196a-5p expression levels and cell viability were significantly increased in the H$_2$O$_2$ + miR-196a-5p mimic group (Fig. 5B and C), whilst the levels of apoptosis, protein expression levels of cleaved caspase-3 and the cleaved caspase-3/caspase-3 ratio were significantly reduced (Fig. 5D-G).

**Discussion**

Age-related cataracts is a type of degenerative disease as a complication of aging, where its pathogenesis is closely associated with cellular senescence and decreased metabolic function in the lens (35). The incidence of age-related cataracts increases with age; 43-54-year-old patients have an incidence of 8.3%, compared with an incidence as high as 70.5% in patients over 75 (14,15). Oxidative stress occurs when the oxidative and antioxidant mechanisms in the body become unbalanced, such that an enhanced oxidative state is favored (36,37). This then promotes inflammatory infiltration by neutrophils, increased secretion of proteases and the production of large quantities of reactive oxidative intermediate products (36,37). Oxidative stress is an adverse effect that is caused by the production and accumulation of free radicals in the body, which is considered to be an important contributing factor to aging and disease (38). It was previously reported that oxidative stress serves an important role in the...
pathogenesis of various types of cataracts (39,40). Age-related cataracts are mainly caused by oxygen free radical-induced damage to LECs, which prompts conformational changes in important proteins, such as E3 ubiquitin-protein ligase Mdm2 and Rho-associated protein kinase 1 in LECs (41,42). To study the role of lncRNA TUG1 in age-related cataracts in vitro, the present study established an in vitro oxidative stress model by exposing the LEC line, SRA01/04, to 200 µM H2O2 for 24 h.

LncRNA TUG1 is expressed in the retina and brain and was discovered to serve an important role in numerous cancer types, including colorectal, esophageal and bladder cancer (43-45). However, to the best of our knowledge, the underlying mechanism of action of TUG1 in age-related cataracts remains to be determined. To investigate the underlying mechanisms of TUG1 in age-related cataracts, the present study predicted and verified the binding site between TUG1 and miR-196a-5p. Through bioinformatics software analysis, it was found that there may be a binding site between miR-196a-5p and TUG1. Therefore, TUG1 may regulate the proliferation and apoptosis of lens epithelial cells by regulating the expression of miR-196a-5p, thereby participating in the occurrence of age-related cataracts. In addition, the expression levels of TUG1 were found to be upregulated, whilst miR-196a-5p expression levels were downregulated, in SRA01/04 cells induced by H2O2. Subsequent transfection experiments revealed that TUG1 negatively regulated miR-196a-5p expression in SRA01/04 cells. However, whether the overexpression of TUG1 has a significant inhibiting effect on miR-196a-5p was not studied in the present study and is a limitation.

To determine the effects of TUG1 on H2O2-induced oxidative damage in SRA01/04 cells and miR-196a-5p expression, cell function experiments were performed in SRA01/04 cells following TUG1 knockdown. Results from the present study revealed that transfection with TUG1-siRNA reduced the H2O2-induced oxidative damage, which was evidenced by the increased cell viability, reduced cell apoptosis, cleaved-caspase3 protein expression and reduced cleaved-caspase3/caspase3 ratios in SRA01/04 cells. By contrast, co-transfection with the miR-196a-5p inhibitor reversed these effects aforementioned. In addition, the overexpression of miR-196a-5p attenuated H2O2-induced oxidative damage in SRA01/04 cells. It was worth mentioning that the apoptosis rate of H2O2 + TUG1-siRNA + miR-196a-5p inhibitor group was similar to that in the H2O2 and H2O2 + control-siRNA group. However, the ratio of cleaved-caspase3/caspase3 in the H2O2 + TUG1-siRNA + miR-196a-5p inhibitor group, was higher compared with that in the H2O2 and H2O2 + control-siRNA group. The reason for this difference between the apoptosis rate and the cleaved-caspase3/caspase3 ratio remain unclear, which require further study.

In conclusion, the findings of the present study revealed that knockdown of lncRNA TUG1 expression protected LECs from oxidative stress-induced apoptosis by increasing miR-196a-5p expression. These results suggest that targeting TUG1 and miR-196a-5p may provide a new therapeutic strategy for patients with age-related cataracts.

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Availability of data and materials

The datasets used and/or generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QS contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. TZ contributed to data collection, statistical analysis and manuscript preparation. QS and TZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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