function to treat acute liver failure in mice through macrophage polarization

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Abstract

Background: Advances in research on stem cell therapy provide new feasible solutions for acute liver failure (ALF) treatment. Recent studies have demonstrated that the expression of hepatocyte nuclear factor 4α (HNF4α) reset within the damaged hepatocytes can restore normal physiological function. This study aimed to determine the role of human umbilical cord mesenchymal stem cells (HuMSCs) overexpressing HNF4α in ALF treatment.

Method: We isolated and cultured HuMSCs in vitro, reversed it by lent virus expression HNF4α (hereinafter referred to as HuMSC-HNF4α). HuMSC-HNF4α was intraperitoneally administrated into the mice immediately after exposed to D-galactosamine / lipopolysaccharide (D-galn / LPS). To investigate their effects in ALF, we performed liver histological and serumbiochemical analysis. Macrophages differentiation and cytokines secreted by HuMSCs were evaluated to elucidate its mechanisms.

Results: We found HuMSC-HNF4α has more obvious therapeutic effects on ALF than the negative control virus transfected the HuMSCs (HuMSC-CON). In vitro, HuMSC-HNF4α promotes the polarization of liver macrophages (Kupffer cells) to M2 phenotype, inhibits the inflammatory response of macrophages and reduces the levels of inflammatory factors such as TNF-α, IL-1β to reduce liver damage.

Conclusion: Our research confirmed that the therapeutic effect of HuMSC-HNF4α on ALF is not the same as the previous passive support but an active intervention on excessive inflammation in the body. This provides new ideas for research and clinical practice in the future.

Keywords: mesenchymal stem cells, hepatocyte nuclear factor 4α, acute liver failure, macrophages

1 Background

Acute liver failure (ALF) is a clinical syndrome characterized by acute and severe liver damage, caused by various factors such as viral infection, toxic drugs, metabolic diseases, and alcohol damage [1]. Orthotopic liver transplantation (OLT) is one of the most effective ways to treat ALF. However, this therapy is limited by the rarity of donor livers and the poor systemic condition of patients before surgery due to long waiting time [2]. Preliminary researches indicate that stem cells play an key role in the treatment of ALF through transdifferentiating into hepatocytes to perform alternative functions, promote liver regeneration, reduce hepatocytes apoptosis, inhibit liver fibrosis, and regulate the immune and inflammatory response [3-6]. Based on importance of immune responses in ALF, the immune regulation of stem cells may be the most important factor in the treatment of ALF [7].

Stem cells, especially mesenchymal stem cells (MSCs), are good vectors for carrying genes due to accessibility, strong differentiation potential, and proliferative capacity. Previous studies have

confirmed that, overexpression of Trx-1, IGF-1, HGF and other genes through reverse transcription and other technologies can enhance the protective effect of MSCs on ALF in different ways [8-10]. MSCs with high expression of CXC chemokine receptor type 4 (CXCR4) can improve the survial and quality of life of patients with ALF caused by small liver syndrome after liver transplantation.

Hepatocyte nuclear factor 4α (HNF4α) is a nuclear transcription factor that has been previously shown to be low expressed in liver diseases such as liver tumors and ALF. The use of lentiviral reverse transcription and other technologies to reset the normal expression of that can restore the normal physiological functions of hepatocytes [11, 12]. Our group has confirmed that overexpression of HNF4α in MSCs though gene transfection technology can promote the differentiation of MSCs into hepatocytes [13, 14]. It is shown that the HNF4α is closely related to various liver diseases such as ALF and has strong regulatory effects on functions of MSCs, suggesting that MSCs with high expression of HNF4α are expected to have better curative effects on ALF.

Macrophages in liver (Kupffer cels) undergo polarized activation to M1 (classical) or M2 (alternative) activation states in response to local microenvironments [15]. M1 macrophages mainly mediate inflammatory damage and promote inflammation. Conversely, M2 phenotype is characterized by anti-inflammatory factors (such as IL-4, IL-10) and protective proteins (TGF-β, VEGF,etc), inhibiting inflammation and repair tissue damage [16, 17]. MSCs have been shown to promote macrophages polarization to M2 phenotype to exert an anti-inflammatory effect by secreting IDO, PGE2, etc. in response to environmental signals including elevated TNF-α and IL-6 [18, 19].

In this study, we established human umbilical cord derived mesenchymal stem cells (HuMSCs) with overexpression of HNF4α (HuMSC-HNF4α) through lentivirus. Their therapeutic effects on D-galactosamine / lipopolysaccharide (D-galn / LPS)-induced ALF were observed, proving a novel therapeutic approach for ALF treatment.

2 Materials and methods

2.1. Isolation and identification of HuMSCs

This study has been approved by the Institutional Review Board and Human Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China. All umbilical cords in this experiment were obtained from healthy full-term cesarean section fetus at Renji Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Written consent for the use of the samples for research purposes was obtained from all patients. We treated HuMSCs as the previous research we have ever published [14]. After induced with conditioned medium for 2-3 weeks, HuMSCs differentiate into chondrogenic (Gibco), osteogenic (Gibco), and adipogenic lineages (Gibco) as indicated by positive type II collagen (Abcam), Alizarin red, and Oil Red O staining respectively. In order to evaluate the phenotypes of HuMSCs, expressioin patterns of CD13, CD90, CD105, CD34, CD45, HLA-DR were detected by flow cytometry (BD Sciences).

2.2. Establishment and identification of HuMSC-HNF4α

Stable overexpression of HNF4α in HuMSCs called HuMSC- HNF4α was constructed according to the previous description of the article published by our group [14]. HuMSCs transfected with a lentiviral vector containing only green fluorescent protein (HuMSC-CON) acted as controls. The lentiviral transduction efficiency was monitored using a confocal laser scanning microscope and blotprotein gel blot analysis. The total protein and mRNA of HuMSC-CON and

HuMSC-HNF4α were used to detect the expression of HNF4α indicated as Western bot and real-time PCR respectively.

2.3. Flow Cytometry

To characterize the phenotypes of HuMSCs and macrophages, flow cytometry (BD Sciences) was performed. Antibodies against the human antigens CD11b, CD68, CD80, CD206, CD13, CD90, CD105, CD34, CD45, HLA-DR were purchased from BD Sciences. The data were analyzed using the CELLQUEST Pro software (BD Sciences).

2.4. Animal

Wild-type mice (C57BL / 6J) were purchased from Shanghai Slark Experimental Animal Co., Ltd., and managed by the Animal Experimental Center of Renji Hospital, Shanghai Jiaotong University (SPF grade). The experimental procedure has been approved by the Animal Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine. 2×10^6 HuMSC-HNF4α and HuMSC-CON were configured into phosphate-buffered saline (PBS) (Invitrogen) solution respectively. Mice aged 5–6 weeks were intraperitoneally injected with HuMSC-HNF4α, HuMSC-CON, and PBS (10 ml/kg). D-GalN (700 mg/kg, Sigma) and LPS (10 μg/kg, Sigma) were administered via abdominal cavity after twenty-four hours of pretreatment. The survival of mice were observed and recorded at 4 h after D-Galn / LPS injection for 48 h. It was recorded every half hour during 4 h to 12 h, and every 6 hours between 12 h and 48 h. At 3 h after intraperitoneal injection of D-Galn / LPS, the mice were anesthetized with 1% pentobarbital sodium. Blood and livers were collected subsequently. Blood samples were centrifuged at 5000rpm for 15min to collect plasma. The plasma and liver tissues were frozen immediately in liquid nitrogen and stored at −80°C until analysis.

2.5. Real-time quantitative reverse transcription PCR

Total RNA was isolated from cultured HuMSCs by TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) and was reverse-transcribed into cDNA using reverse transcription kit (TaKaRa). A PCR reaction was carried out in 20 μl of a final volume containing 0.1 μM of each forward and reverse primer, cDNA and 10 μl SYBR Green PCR Master Mix (TaKaRa). Amplification was conducted and analysed using an StepOnePlus real-time PCR machine (ABI).

2.6. Western blot

To determine the expression of HNF4α, total protein was lysed with RIPA peptide lysis buffer (Beyotime Biotechnology, Jiangsu, China) containing 1% protease inhibitors (Pierce). Western blot analysis were performed according to anti-β-actin (Santa Cruz) and anti-HNF4α (Santa Cruz).

2.7. Detection of serological indicators

ALT and AST in plasma were ditected with an automatic biochemical detector. ELISA kits (R&D) were used to evaluate TNF-α and IL-1β in plasma. HuMSC-CON and HuMSC-HNF4α were seeded in 6-well plates at a density of 5×10^5 / well. When the cells reached 70% confluence, remaining supernatant was washed with PBS. Serum-free F12 (Gibco) was used to continue culture routinely. After 48 h, the supernatant was collected into 15 ml centrifuge tubes and tested with ELISA kits to assess the levels of various immune factor indicators such as IL-10, M-CSF.

2.8. Histopathological examination

Tissue samples were cut into appropriate size and fixed with 4% paraformaldehyde for H&E and immunohistochemical. Antibodies for immunohistochemical were used as follows: F4 / 80 (Abcam), MPO (Abcam), TNF-α (Abcam). TUNEL was performed for apoptosis.

2.9. THP-1 cell culture and differentiation into macrophages

The medium of THP-1 is 1640 complete medium (Gibco) with 1% double antibody and 10% FBS. THP-1 cells with good growth activity are seeded in six-well plates at a density of 1×10^6 / well. PMA (sigma) is added to the medium to induce differentiation at a final concentration of 100 ng/ml. The medium was changed at 24 h after induction and cells were cultured for 4 days. Following discarding the medium of induced macrophages, the plates were washed for 3 times with PBS. Fresh culture medium was used to observe the morphology of cells under a light microscope. The induced macrophages were collected and the surface antigens CD11b and CD68 of cells were detected by flow cytometry. THP-1 cells were induced to differentiate into macrophages and cultured in six-well plates. A 0.4 μm-sized Transwell chamber (Corning) was placed on the six-well plate. 5×10^5 HuMSC-HNF4α was added to the chamber (control group with HuMSC-CON, negative control group without cells), and co-cultured with macrophages in 1640 medium for 24 h to construct co-culture systems. The MTT was used to detect the viability of macrophages. There being no significant difference in cell viability between the treatment group and the control group, disturbing factors could be excluded. After collecting cells of the three groups, the Transwell chamber was discarded. The mixed cells were cultured and stimulated in 1640 medium containing LPS (1 μg/ml) for 6 h following medium change. The negative control group continued to be cultured with normal 1640 medium 6 h. Cell culture medium of macrophages from the three groups was collected. Immune factors secreted by macrophages such as TNF-α and IL-1β were detected by ELISA kit. Expression patterns of macrophages CD80 and CD206 were detected by flow cytometry.

2.10. Statistical analysis

The data of this subject were expressed as mean ± standard deviation (mean ± SD). SPSS 19.0 statistical software was used for analysis. The comparison of indicators depended on single factor analysis of variance. *T* test was used for comparison between groups. $P < 0.05$ was considered as statistical significance level.

3 Results

3.1 Identification of HuMSCs and HuMSC-HNF4α

Fibroblast-like cells isolated from the umbilical cord were spindle-shaped polygonal, and grew adhering to the wall (Fig.1a). HuMSCs differentiated into chondrogenic, osteogenic, and adipogenic lineages as mentioned. The morphological changes were consistent with the performance of differentiation indicated by Oil Red O staining, Alizarin red, and type II collagen (Fig.1b, c, d). Flow cytometry revealed that the cells overexpressed CD73, CD90, and CD105, but expressed CD34, CD45, and HLA-DR lowly (Fig.1e). In summary, the cell met the identification criteria of MSCs [20].

HuMSCs were transfected with either pWIPIGFP (HuMSC-CON) or pWIPI-HNF4α-GFP (HuMSC-HNF4α) lentiviral vector for 5 to 7 days. After infection, the ratio of GFP-positive cells revealed that transfection efficiency was > 95% (Fig.2a). Western blot and real-time PCR indicated the obvious expression of HNF4α in HuMSC-HNF4α (Fig.2b). In summary, HuMSC-HNF4α and HuMSC-CON were successfully constructed.

3.2 Protective effects of HuMSC-HNF4α in mice with ALF

Survival curves were drawn according to a 48-hours survival study after D-Galn / LPS injection. (Fig. 3a). Intraperitoneal injection of HuMSC-HNF4α and HuMSC-CON could improve the survival of mice with ALF. The HuMSC-HNF4α group had 44% mortality on hour 9, whereas HuMSC-CON group had 90% mortality on hour 9. HuMSC-HNF4α performed a better protective

effect than HuMSC-CON as shown by prolonging survival. The differences among groups were statistically significant ($P < 0.05$).

The plasma levels of liver enzyme indexes ALT and AST detected in LPS / D-GalN-induced ALF mouse model significantly reduced in HuMSC-HNF4α and HuMSC-CON group, compared to PBS group ($P < 0.01$). The efficacy of HuMSC-HNF4α was better (Fig.3b).

We then examined the levels of pro-inflammatory cytokines in circulation. TNF-α and IL-1β plasma levels were both significantly reduced in HuMSC-HNF4α and HuMSC-CON mice after ALF. However, the levels of TNF-α and IL-1β in HuMSC-HNF4α mice were lower (Fig.3c). We found statistical significance among groups ($P < 0.01$). It was suggested that HuMSC-HNF4α inhibited inflammation to a certain digree.

H&E revealed extensive congestion and necrosis of liver tissues in liver tissues of PBS group. The liver tissues of HuMSC-CON group exhibited moderate microscopic deterioration. In contrast, only mild liver injury was seen in liver tissues of HuMSC-HNF4α group (Fig.3d). HuMSC-HNF4α alleviated liver damage in mice with ALF. The number of TUNEL-positive cells in the liver tissues significantly inreased in PBS group compared to other groups. The mice injected with HuMSC-HNF4α had a lowest ratio of apoptosis (Fig.4a).

The levels of F4 / 80, MPO, and TNF-α indicated liver inflammatory status. F4 / 80 is a specific index of macrophages. Liver sections from HuMSC-HNF4α mice contained fewer F4 / 80-positive cells (macrophages and Kupffer cells) compared with those of HuMSC-CON mice. A large number of macrophages infiltrated in liver tissues of mice injected with PBS (Fig.4b). TNF-α, secreted by activated macrophages, is the most important inflammatory factor that causes hepatocyte apoptosis and necrosis during the pathogenesis of ALF. The level of TNF-α in liver tissues of mice injected by HuMSC-HNF4α was lower than that in HuMSC-CON group (Fig.4b). MPO is a specific indicator of neutrophils in mice. There was no significant difference of neutrophil infiltration in liver tissues among these three groups (Fig.4b). Taken together, these results indicated that HuMSC-HNF4α mainly acted on macrophages. Experiments were subsequently designed to explore specific mechanism of macrophages action in vitro.

3.3 HuMSC-HNF4α inhibits LPS-stimulated macrophage inflammation and promotes macrophages to polarize to M2 states

Circular and suspended THP-1 cells were cultured with PMA (100ng/ml) for 24 h, the cells grew adherently and lost the ability to proliferate subsequently. After incubated for 4 days, the cells became larger and extended amoeba-like pseudopodia (Fig.5a). Compared to undifferentiated cells, expression of human macrophage markers CD11b and CD68 were significantly higher (Fig.5b). In summary, the cells met the criteria of experimental macrophages [21].

Macrophages co-cultured with HuMSC-HNF4α or HuMSC-CON or no cell were stimulated by LPS for 6 h. The culture supernatant was collected and determined the levels of TNF-α and IL-1β. Macrophages released a large number of inflammatory factors such as TNF-α and IL-1β after stimulation. Either HuMSC-HNF4α or HuMSC-CON decreased the levels of inflammatory factors secreted by macrophages. HuMSC-HNF4α inhibited the release of TNF-α more significantly ($P < 0.01$). As for the effect of inhibiting the release of IL-1β, there was no difference between HuMSC-HNF4α and HuMSC-CON (Fig.5c).

Macrophages polarized towards M1 phenotype or M2 phenotype according to the local microenvironment. M1 macrophages are characterized byoverexpression of CD80, CD86, etc. M2 penotype overexpresses CD206, CD369 (Dectin-1), etc [22, 23]. After collecting macrophages co-

cultured with HuMSC-HNF4α or HuMSC-CON or no cell stimulated by LPS for 6 h and macrophages without LPS (CTR), CD80 and CD206 were examined by flow cytometry. The expression of CD80 in macrophages co-cultured with HuMSC-HNF4α decreased. In contrast, the level of CD206 increased (Fig.5d). Such results indicated that HuMSC-HNF4α promoted M2 polarization of macrophages, which also consisted with the previous conclusions that HuMSC-HNF4α reduced the level of TNF-α secreted by macrophages.

3.4 Overexpression of HNF4α up-regulated the level of IL-10 and M-CSF secreted by HuMSCs

In order to find the factors secreted by HuMSC-HNF4α that affected the polarization of macrophages, the culture supernatant of HuMSC-HNF4α and HuMSC-CON were collected for protein chip analysis (Fig.6a). The results showed that overexpression of HNF4α was able to change the levels of various immune factors secreted by HuMSCs. To screen out the immune factors closely related to macrophages, the culture supernatant of HuMSC-HNF4α and HuMSC-CON was taken to examine IL-10 and M- CSF. The levels of them from HuMSC-HNF4α were significantly higher than those of HuMSC-CON ($P < 0.01$) (Fig.6b). Recent literatures have confirmed that IL-10 and M-CSF can promote macrophages to polarize towards M1 phenotype and play a role in inhibiting inflammation and repairing damage [24, 25]. It is inferred that HNF4α may enhance the immunoregulatory function of HuMSCs by up-regulating the secretion of immune factors such as IL-10 and M-CSF.

4 Discussion

In the past, the treatments of ALF have focused on passive support and functional alternative therapy. Hepatocyte transplantation or bioartificial liver, defined as alternative treatment, aims to transplant exogenous hepatocytes into the patient's body to replace partial function of liver and support the anabolic needs of body. The source of hepatocytes is generally immortalized hepatocytes [26, 27]. However, passive support of hepatocytes alone is not enough to reverse ALF. Scholars have tried to transplant other cells to enhance the therapeutic effects. Zheng et al.co-microencapsulated rat hepatocytes and sertoli cells and transplanted them into rats with ALF. Co-microcapsules of these two cells could significantly enhance the metabolic function of hepatocytes and obtain significant results in the treatment of ALF [28].

With the rapid development of genetic engineering technology, carrying certain genes to enhance the therapeutic effect of cells has replaced the use of existing cells to treat diseases. Inspired by these studies, we have also committed to gene-regulated MSCs transplantation for the treatment of ALF in order to enhance the therapeutic effect of MSCs recently.

Our group has confirmed that overexpressed HNF4α in MSCs can promote MSCs to differentiate into hepatocytes in previous studies through gene transfection technology [10, 14]. These studies have shown that the HNF4α, conducting strongly regulatory effects on various functions of MSCs, is closely related to various liver diseases such as ALF. MSCs with overexpression of HNF4α are expected to have better therapeutic effect on ALF. In this study, we designed a corresponding experiment to explore the therapeutic effect and mechanism of HuMSC-HNF4α on ALF. In vivo, HuMSC-HNF4α effectively improved the survival of mice with ALF, suppressed the immune responses, reduced liver damage, and reduced necrosis and apoptosis of liver tissues. It had been verified from various aspects such as serology, histology and cytology. The therapeutic effect of HuMSC-HNF4α on ALF is to inhibit the inflammatory response of liver macrophages, reduce the infiltration of macrophages in liver tissues, and reduce the release of

inflammatory factors.

Based on the important role of macrophages in the pathogenesis of ALF, this study designed an experiment to co-culture HuMSC-HNF4α and macrophages to explore the effect of HuMSC-HNF4α on the inflammatory response of macrophages in vitro. We have also confirmed that HuMSC-HNF4α inhibited the inflammatory responses caused by LPS-stimulated and reduced the level of TNF-α secreted by macrophages.

Researchers have found that HuMSC-HNF4α promoted macrophages polarization towards M2 phenotype, which suggested that HuMSC-HNF4α changed the microenvironment of the culture medium. In order to find the factors secreted by HuMSC-HNF4α influencing the polarization of macrophages, we collected the culture supernatant of HuMSC-HNF4α and HuMSC-CON for protein chip analysis to examine the distinguished factors. More than 40 factors perfomed significantly significance were found ($P < 0.05$). Factors related to the inflammatory response of macrophage were examined to confirm that the levels of IL-10 and M-CSF secreted by HuMSC-HNF4α were significantly higher that of HuMSC-CON. IL-10 and M-CSF can activate the polarization of macrophages towards M2 phenotype, and inhibit inflammation and repairing damage [24, 25]. Thus, we speculated that HNF4α enhanced the immune regulatory function of HuMSCs by up-regulating the secretion of immune factors such as IL-10 and M-CSF.

Recent studies have confirmed that immunoinflammatory responses played an important role in ALF so that the regulatory of MSCs on immunity has become the latest focus. Shalom et al. conducted a global gene expression analysis of human bone marrow-derived MSCs. They found that the typical pro-inflammatory factor TNFα prominently induced the expression of the pro-inflammatory mediators CCL2, CXCL8 (IL-8), and cyclooxygenase-2 (Cox-2) in MSCs through the NF-κB / p65 pathway [29]. Higashimoto et al. pointed out that adipose-derived MSCs mainly inhibited CD4 + T cells and CD11b + myeloid cells to exert anti-inflammatory effects, such as reducing the secretion of TNF-α and CXCL10 [30]. Zhang et al. found that MSCs could induce the differentiation of CD11c + B220 - DC precursors into Regulatory Dendritic Cells (RDCs). RDCs can suppress the immune response of T cells and induce T-regulatory cell (Treg) differentiation through the secretion of TGF-β so that RDCs protected the bodies from fulminant liver failure [31]. In ALF caused by different etiologies, MSCs exerted immunoregulatory functions on lymphocytes, NKT cells, DC cells and other immune effector cells. Similarly, we also found that HuMSC-HNF4α had a strong anti-inflammatory effect on macrophages in ALF. It reminded us that both immunoregulatory function and effctors of MSCs were complicated. There are still unknown mechanism waiting to be explored.

HuMSC-HNF4α with powerful immunomodulatory effect in the pathogenesis of ALF is expected to form a mixed system with immortalized hepatocytes to jointly transplant or construct a hybrid biological artificial liver. This process combines functional support and immune regulation to treat ALF more effectively. Our group also regards this as future research direction. However, this study also had certain deficiencies. After protein chip, the mechanism needs to be further explored in vivo, which will be improved in the future.

5 Conclusions

This project has confirmed the therapeutic effects of HuMSC-HNF4α in ALF in vivo and in vitro. It revealed that HuMSC-HNF4α promoted macrophages to polarize towards M2 phenotype by secreting IL-10 and M-CSF. HuMSC-HNF4α suppresses inflammation and repairing damage,

which actively interferes with excessive inflammatory reactions in the body instead of passive support. Meanwhile, it provides new ideas for scientific research and clinical.

Abbreviations

HuMSCs, human umbilical cord mesenchymal strem cells; MSCs, mesenchymal strem cells; HNF4α, hepatocyte nuclear factor 4α; ALF, acute liver failure; HuMSC-HNF4α, HuMSCs overexpressing HNF4α; HuMSC-CON, HuMSCs without HNF4α; PBS, phosphate-buffered saline; D-Galn, D-Galactosamine; LPS, Lipopolysaccharide; OLT, orthotopic liver transplantation

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Authors' contributions

YPY, QQZ, QX, and HHL were involved in conception and design of the study. NW and QQZ performed the in vivo experiment. YPY and QQZ performed the in vitro experiments. JC and LX performed the data analysis and interpretation. YPY, JJZ, and HLH wrote the manuscript. NW, QX, JZ, JJZ, and HLH were involved in review and editing of the manuscript. All authors commented the final version. All authors had full access to all data. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials about this work are available.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board and Human Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.

Consent for publication

All authors consent to publish this manuscript. For care and use of animals and were followed.

Competing interests

The authors declare that they have no competing interests.

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