



FULL LENGTH ARTICLE

Endothelial DLC1 is dispensable for liver and kidney function in mice

Ying Tan, Su Hao Lo*

Department of Biochemistry and Molecular Medicine, University of California-Davis, Sacramento, CA 95817, USA

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Abstract DLC1 is a focal adhesion molecule that regulates cell polarity, proliferation, migration, and survival. DLC1 functions as a tumor suppressor and its expression is often down-regulated in various malignant neoplasms of epithelial origin. Recent studies have suggested that lack of DLC1 in endothelial cells may contribute to the development of angiosarcoma, and that DLC1 mutations have been identified in patients with nephrotic syndrome, a disease mainly due to leaky glomerular filtration barriers. To demonstrate whether lack of endothelial DLC1 induces angiosarcoma and/or damages glomerular capillaries leading to nephrotic syndrome, we have extended our analyses on endothelial cell-specific DLC1 knockout mice with focuses on their liver and kidney function. Mice were monitored up to 24 months of age. However, no histological or clinical difference was found between DLC1 knockout and wild type mice, indicating that lack of endothelial DLC1 alone does not compromise kidney and liver function in mice.

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Introduction

DLC1 (deleted in liver cancer 1) is a tumor suppressor gene that is originally identified in hepatocellular carcinoma¹ and then in a variety of cancer types, such as lung, colon, prostate, breast, kidney and brain.^{2–6} In cancer cells, its

expression is often lost or down-regulated due to genetic or epigenetic modifications, and re-expression of DLC1 suppresses cancer cell proliferation, migration, and colony formation.^{2–6} DLC1 is expressed in most tissues and DLC1 total knockout mice die by E10.5,⁷ demonstrating its essential role during embryogenesis. However, the function of DLC1 in adult tissues are not well understood.

Recent studies have shown that DLC1 deficiency may play a critical role in the development of angiosarcoma,^{8,9} which is a rare yet highly malignant soft tissue sarcoma of endothelial cell origin. Angiosarcoma may derive from the endothelial layer of blood or lymph vessels and can occur at anywhere in the body but most of them arise in skin, breast and liver.¹⁰ Hepatic angiosarcoma is also uncommon among

* Corresponding author. Department of Biochemistry and Molecular Medicine, University of California-Davis, 4635 Second Ave. Research I, Room 3210, Sacramento, CA 95817, USA.

E-mail address: shlo@ucdavis.edu (S.H. Lo).

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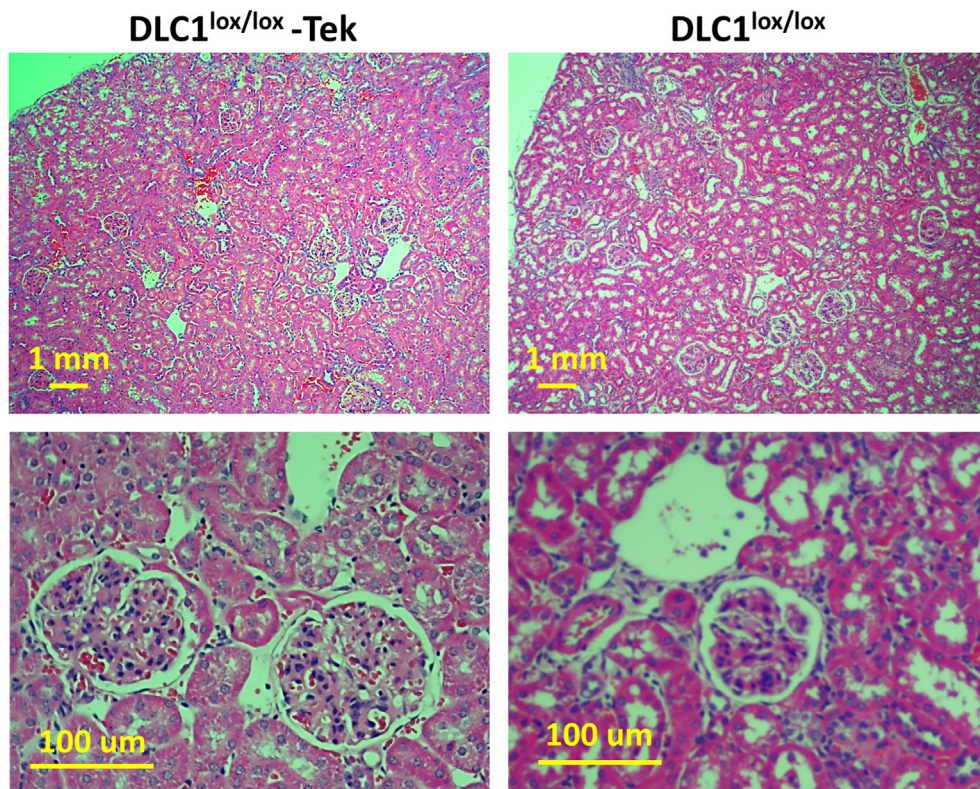


Figure 1 Histological evaluations of kidneys from $\text{DLC1}^{\text{lox/lox-Tek}}$ and control mice. Representative H&E staining images of kidney sections from 24 months old $\text{DLC1}^{\text{lox/lox-Tek}}$ or $\text{DLC1}^{\text{lox/lox}}$ mice. Glomeruli, tubules and blood vessels are well organized (upper panel) and their structures appear to be normal (lower panel) in mutant and control kidneys.

all primary hepatic malignancies yet it is the most frequent malignant mesenchymal cancer of the liver.¹¹ The pathogenesis and risk factor of angiosarcoma are currently unclear.

Nephrotic syndrome (NS) is one of the most common causes of glomerular disease¹² and is characterized by albuminuria, hypoalbuminemia, hyperlipidemia, and edema.¹² It may lead to chronic kidney disease that results in end-stage renal failure requiring dialysis or renal replacement for survival. Nephrotic syndrome is the result of a leaky glomerular filtration barrier, which is consisting of a three-layer structure of endothelial cells, glomerular basement membrane (GBM), and podocytes. Dysfunction of any of these layers will compromise the glomerular filtration barrier and lead to nephrotic syndrome. DLC1 mutations were recently identified as the potential cause of nephrotic syndrome in several patient families.¹³ However, their direct involvement in the NS development remains to be demonstrated.

Here, we use endothelial cell-specific DLC1 knockout mice to examine the roles of DLC1 in the kidney and liver structure/function with focuses on nephrotic syndrome and hepatic angiosarcoma development.

Materials and methods

Mouse analysis

Endothelial cell-specific DLC1 knockout mice were generated by crossing $\text{DLC1}^{\text{lox/lox}}$ from our laboratory with B6.Cg-Tg

($\text{Tek-cre}^{\text{1Ywa/J}}$) from Jackson laboratory as described.¹⁴ These mice were maintained under a specific pathogen-free facility at the University of California–Davis. All mouse related handlings were performed with protocols approved by the animal ethics committee at our University. Blood and urine were collected from mice at various ages. Liver and kidney function chemical panel measurements were conducted at the Comparative Pathology Laboratory at UC Davis. Kidneys and livers were collected, fixed, sectioned, and stained with hematoxylin and eosin for histological evaluation. Urine samples were analyzed by SDS-PAGE and Bio-Safe Coomassie G-250 stain to detect albuminuria.

Immunohistochemistry

Tissue sections were treated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Slides were then incubated at 4°C overnight with rabbit anti-mouse CD31 (#77699, Cell Signaling Technology, Danvers, MA USA) at 1:100 dilution. Signal was detected with Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) and developed with diaminobenzidine substrate. Slides were counterstained with hematoxylin.

Isolation of mouse endothelial cells

Primary endothelial cells were isolated from $\text{DLC1}^{\text{lox/lox-Tek}}$ and $\text{DLC1}^{\text{lox/lox}}$ mice as previously described.¹⁴

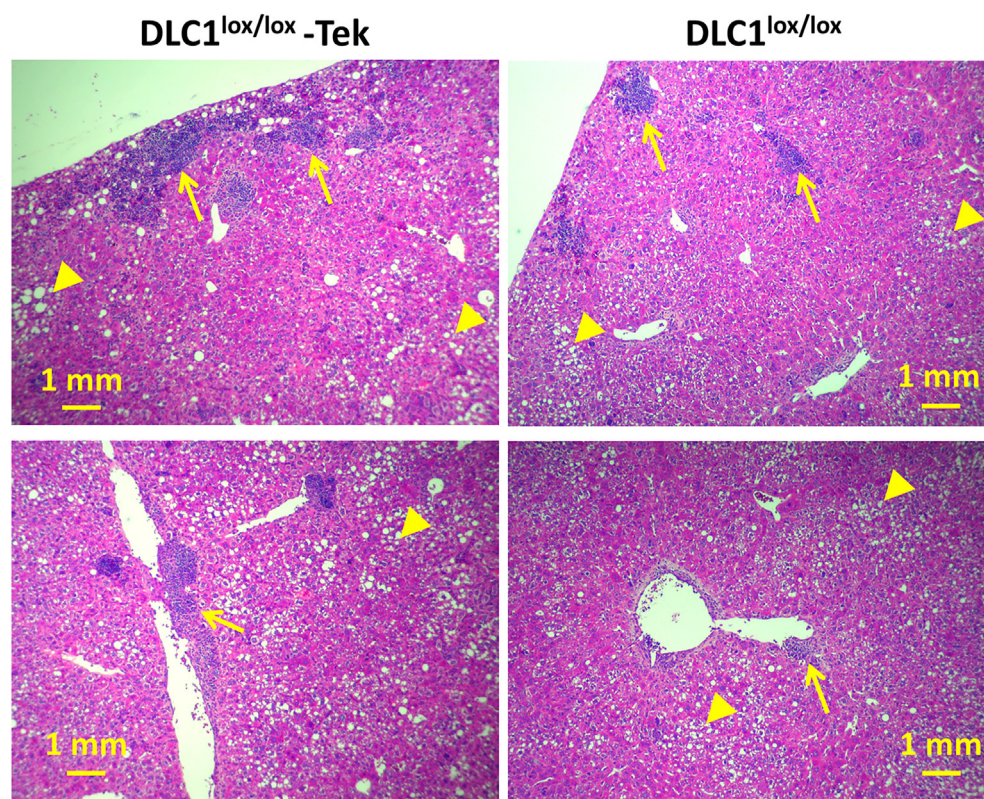


Figure 2 Histological evaluations of livers from $\text{DLC1}^{\text{lox/lox}}$ -Tek and control mice. Representative H&E staining images of liver sections from 24 months old $\text{DLC1}^{\text{lox/lox}}$ -Tek or $\text{DLC1}^{\text{lox/lox}}$ mice. Inflammation infiltrates (arrows) and macrovesicular fatty changes (arrowheads) are observed in mutant and control livers.

Statistics

Data were presented as the mean $\pm$ SD. Student's *t* test was used for statistical analysis of the difference between two groups. All statistical tests were two-tailed and *P* values < 0.05 were considered statistically significant.

Results and discussion

Since recent studies have reported that DLC1 mutations were identified as the potential cause of NS¹³ and dysfunctional blood vessels in the glomeruli might lead to albuminuria,¹⁵ a major feature of NS, and that DLC1 deficiency is implicated in the development of angiosarcoma^{8,9} and the liver is one of the frequent sites of angiosarcoma,¹⁰ we decided to investigate the effects of endothelial DLC1 deficiency on the kidney and liver structure/function using $\text{DLC1}^{\text{lox/lox}}$:Tek-Cre ($\text{DLC1}^{\text{lox/lox}}$ -Tek) mice. We had previously examined the $\text{DLC1}^{\text{lox/lox}}$ -Tek mice and reported that their angiogenesis processes are compromised as demonstrated by gel plug and aortic ring sprouting assays.¹⁴ In the current study, mice were further monitored up to 24 months of age. However, we did not observe any noticeable difference between $\text{DLC1}^{\text{lox/lox}}$ -Tek and $\text{DLC1}^{\text{lox/lox}}$ mice on their overall gross appearance, behavior, and health under the specific pathogen-free (SPF) living condition. Histologically, well organized renal structures were presented in kidneys from

both $\text{DLC1}^{\text{lox/lox}}$ -Tek and control mice at 24 months of age (Fig. 1). Glomerular, tubular and vascular structures appear to be normal (Fig. 1). Mild interstitial infiltrates were occasionally detected in $\text{DLC1}^{\text{lox/lox}}$ -Tek and $\text{DLC1}^{\text{lox/lox}}$ kidneys (not shown). In livers, mild lipodosis and inflammatory cell infiltrates were observed in both $\text{DLC1}^{\text{lox/lox}}$ -Tek and $\text{DLC1}^{\text{lox/lox}}$ mice likely due to aging (Fig. 2). Blood vessels were indistinguishable between $\text{DLC1}^{\text{lox/lox}}$ -Tek and $\text{DLC1}^{\text{lox/lox}}$ samples (Fig. 2). The distribution patterns of CD31 positive endothelial layers in livers and kidneys were very similar between $\text{DLC1}^{\text{lox/lox}}$ -Tek and $\text{DLC1}^{\text{lox/lox}}$ mice by immunohistochemistry staining (Fig. 3A). Because anti- DLC1 antibodies were not able to detect mouse endogenous DLC1 by immunohistochemistry staining, we had isolated primary endothelial cells from mice and confirmed the lack of DLC1 expression in endothelial cells from $\text{DLC1}^{\text{lox/lox}}$ -Tek mice by immunoblotting analysis (Fig. 3B). Clinically, blood tests for liver and kidney function were shown in Figure 4. No statistically significant difference was found in all 8 markers between $\text{DLC1}^{\text{lox/lox}}$ -Tek and $\text{DLC1}^{\text{lox/lox}}$ mice. However, if one-tailed, instead of two-tailed, distribution was applied, the *p* values of serum alanine transaminase, aspartate transaminase, albumin, BUN, and creatinine levels were lower than 0.05, showing statistical significance. Nonetheless, the levels of these markers in $\text{DLC1}^{\text{lox/lox}}$ -Tek mice were still within the ranges of normal liver/kidney functions. Albuminuria was not detected even in 24 months old $\text{DLC1}^{\text{lox/lox}}$ -Tek mice (Fig. 3C), whereas albuminuria was

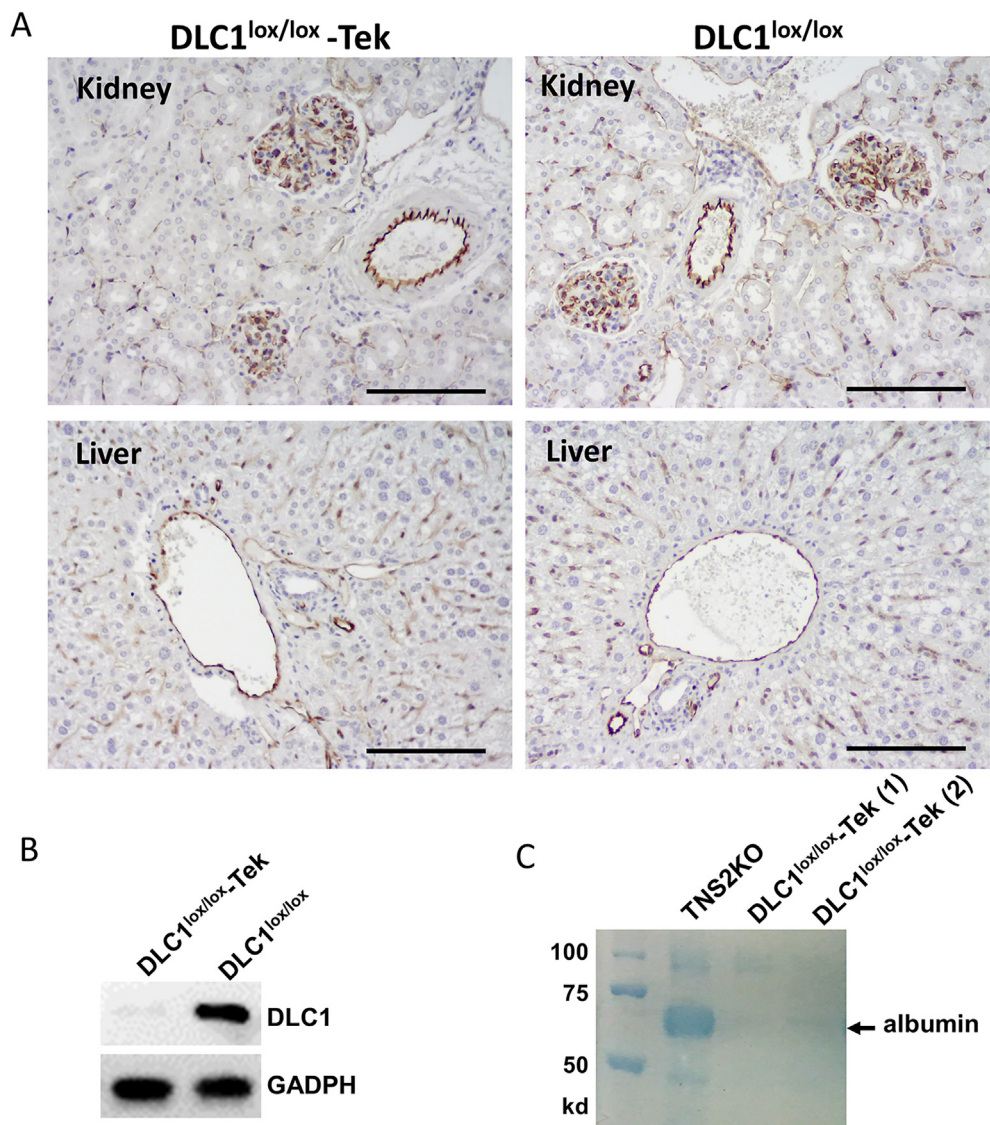


Figure 3 Endothelial DLC1 deficiency does not alter blood vessel distributions or cause albuminuria. (A) kidney and liver sections of 24 months old $DLC1^{lox/lox}$ -Tek or $DLC1^{lox/lox}$ mice were immunostained with CD31 antibody. Scale bar = 100 μ m. (B) Cell lysates of primary endothelial cells isolated from $DLC1^{lox/lox}$ -Tek or $DLC1^{lox/lox}$ mice were immunoblotted with DLC1 or GAPDH antibodies. (C) Urine samples (5 μ l) from a 10-week old TNS2 knockout mouse (in the FVB background) and two 24-month old $DLC1^{lox/lox}$ -Tek mice were analyzed by a SDS-PAGE gel.

prominent in a 10 weeks old TNS2 knockout mouse, a nephrotic syndrome mouse model.¹⁶ Altogether, lack of endothelial DLC1 does not compromise kidney and liver function, and shows no sign of nephrotic syndrome or hepatic angiosarcoma.

Although the functions of DLC1 in tumorigenesis (especially in carcinoma) have been well recognized, the critical roles of DLC1 in endothelial cells are only revealed recently. These findings include (1) knockout or depletion of DLC1 promotes endothelial cell migration and reduces tube formation activities¹⁴; (2) down-regulation of DLC1 enhances cell survival, cell cycle and reduces contact inhibition of growth in confluent endothelial cells⁹; (3) silencing of endothelial DLC1 reduces cell stiffness and mimicked leukocyte transmigration kinetics¹⁷; (4) depletion of endothelial DLC1 disrupts cell polarization in directed

collective migration and inhibits the formation of angiogenic sprouts.¹⁸ These findings are mainly concluded from analyses of DLC1-silenced human umbilical vein endothelial cells (HUVECs). However, our previous¹⁴ and current studies using endothelial cell-specific DLC1 knockout mice have demonstrated that endothelial DLC1 is not absolutely required for embryogenesis, liver and kidney function, mouse survival as well as preventing angiosarcoma formation. It is likely that additional "hit(s)" are needed to induce angiosarcoma and our $DLC1^{lox/lox}$ -Tek mouse line will be a useful animal model for identifying and validating the candidates.

While our study demonstrates that endothelial DLC1 is not required for normal renal function, this study does not rule out the potential involvement of DLC1 in nephrotic syndrome through DLC1's role in the podocyte, which is

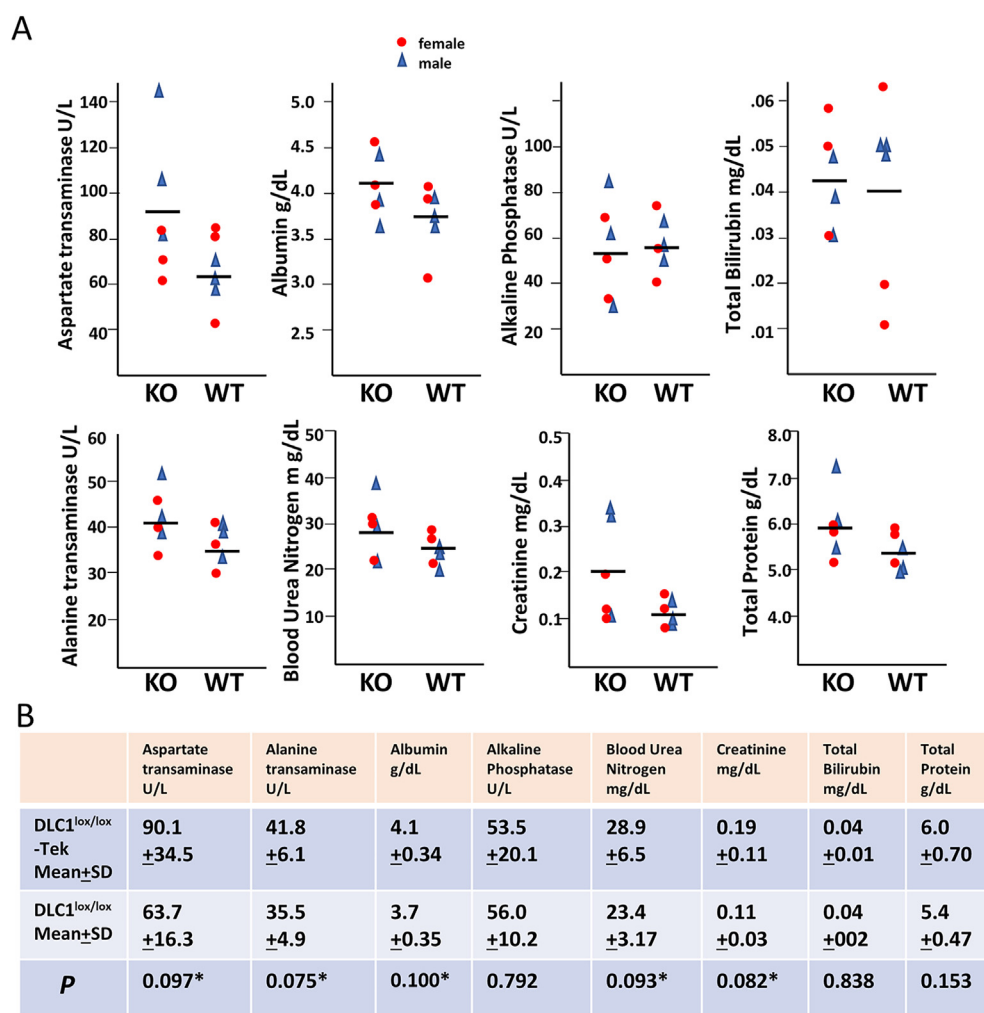


Figure 4 Summary of liver and kidney functional tests. Blood samples from 24 months old DLC1^{lox/lox}-Tek (KO) or DLC1^{lox/lox} (WT) mice ($N = 6$ each) were analyzed for indicated markers. Data were presented as scatter plots (A) and a table of the mean $\pm$ SD (B). P -values shown were calculated using two-tailed t test with equal variance. * These P -values < 0.05 under one-tailed t test with equal variance. Red dot: female, blue triangle: male.

another important cell type in the glomerulus. Podocytes adhere to the GBM through their foot processes assembling cell-matrix junction and establishing intercellular junctions that form slit diaphragm filtration barrier, which is essential for normal renal function. Because podocytes have very limited, if any, regenerative capacities, damaged cells are replaced by healthy podocytes migrating to the injury site.¹⁹ Dysregulated focal adhesions and/or cell-cell junctions will impair podocyte function and repair. For example, lack of or mutated α -actinin-4 focal adhesion protein in podocytes leads to progressive proteinuria and glomerular disease.^{20,21} Therefore, we are in the process of generating and analyzing podocyte-specific DLC1 knockout mice to understand the role of DLC1 in podocytes relating to nephrotic syndrome.

Conflict of interests

The authors disclose no potential conflicts of interest.

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