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β4 integrin is not essential for localization of hemidesmosome proteins plectin and CD151 in cerebral vessels

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Abstract:

Objective: In the central nervous system (CNS), β4 integrin is predominantly expressed by endothelial cells lining arterioles. As β4 integrin plays an essential role in epithelial tissues, organizing structural proteins into specialized adhesive structures called hemidesmosomes (HD), the aim of this study was to determine whether it plays a similar role in CNS endothelium. **Methods:** Dual-immunofluorescence was used to examine the relationship between β4 integrin expression and co-expression of the HD proteins plectin and CD151 in frozen sections of mouse brain, both under normoxic (control) conditions and following chronic mild hypoxia. The requirement of β4 integrin for the localization of HD proteins was examined in transgenic mice lacking β4 integrin expression specifically in endothelial cells (β4-EC-KO mice). **Results:** Immunofluorescence revealed that in the normal adult CNS, plectin and CD151 strongly co-localized with β4 integrin in arterioles. However, in the chronic mild hypoxia model, in which extensive cerebrovascular remodeling is observed, plectin and CD151 were strongly upregulated on all cerebral vessels, but surprisingly, in capillaries, this occurred in a β4 integrin-independent manner. Unexpectedly, absence of endothelial β4 integrin (in β4-EC-KO mice) had no impact on the expression level or distribution pattern of plectin and CD151 within stable or remodeling cerebral vessels. **Conclusions:** These results demonstrate that the HD proteins plectin and CD151 are closely associated with β4 integrin on arterioles in normal brain, and are strongly upregulated on remodeling blood vessels. However, unlike its described role in the epidermis, β4 integrin is not essential for localization or regulation of expression of plectin and CD151 in cerebral vessels.

Key words:

CD151, central nervous system, hemidesmosome, integrin, plectin, vessel

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Introduction

Hemidesmosomes (HDs) are specialized adhesive structures that promote epithelial attachment.^[1,2] They contain multiple proteins, including α6β4 integrin, tetraspanin CD151, two plakins family members, plectin, and BP230, as well as Type XVII collagen BP180.^[3] HD plays an essential role in tissues exposed to high mechanical forces by establishing a strong mechanical link between laminin in the extracellular matrix and intracellular cytoskeletal intermediate filament proteins, such as keratin or vimentin.^[4] The importance of HD in conferring structural integrity is highlighted by the findings that mutations in HD genes in humans or genetic deletions in mice result in skin blistering disorders of varying severity. In particular, genetic deletion of the α6 or β4 integrin subunits

in mice results in a perinatal lethal phenotype caused by defective epidermal integrity.^[5,6] This leads to a severe skin blistering condition, akin to the human condition, junctional epidermolysis bullosa (JEB). Consistent with this, mutations in the β4 integrin gene are a leading cause of JEB.^[7]

Structurally, the β4 integrin subunit is unique. Many integrin β subunits, including β1, β3, and β5, show strong homology within the short cytoplasmic domain (~50 amino acid) and sequences within this domain interact with cytoplasmic adaptor proteins to form transmembrane links with the actin cytoskeleton.^[8] In contrast, the cytoplasmic domain of β4 integrin is twenty times longer than other β subunits (more than 1000 amino acids).^[9] It also contains sequences that interact not with the actin cytoskeleton, but with intermediate cytoskeletal proteins, such as keratin and vimentin. Aside from its role in maintaining

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epithelial integrity, not much is known about the function of $\beta 4$ integrin. This is surprising in light of the fact that $\beta 4$ integrin shows very specific expression patterns within several tissues, including vascular system. In the central nervous system (CNS), $\beta 4$ integrin is expressed by a specific subset of blood vessels.^[10] Until recently, it was unclear which type of cerebral vessel or which vascular cell type expresses $\beta 4$ integrin. It had previously been suggested that $\beta 4$ integrin was expressed by astrocyte end-feet that run along cerebral blood vessels.^[10,11] However, in a series of cell type-specific gene deletion experiments, in which $\beta 4$ integrin was deleted from either endothelial cells or astrocytes, we recently demonstrated that in cerebral vessels, $\beta 4$ integrin is expressed specifically by endothelial cells lining arterioles and is not expressed by astrocytes or smooth muscle cells.^[12] Interestingly, during neuroinflammation, $\beta 4$ integrin expression expands to a greater number of vessels and expression levels per vessel are markedly upregulated.^[13]

Previous studies have identified HD structures within the CNS,^[14] as well as the expression of several HD protein components, including $\beta 4$ integrin,^[10,11] CD151,^[15] and plectin^[16,17] within the brain. However, while it is well established that in epithelial tissues, $\beta 4$ integrin plays an essential role in assembling HD by orchestrating localization of the different protein components,^[6,18] it has yet to be determined whether $\beta 4$ integrin plays a similar role in the CNS. To this end, the goal of this project was to define the relationship between $\beta 4$ integrin and HD proteins in the CNS by: (i) defining the expression pattern and degree of colocalization of $\beta 4$ integrin with the HD proteins plectin and CD151 in the normal CNS, (ii) determining how $\beta 4$ integrin expression and degree of colocalization are altered during hypoxic-induced vascular remodeling, and (iii) determining whether the absence of endothelial $\beta 4$ integrin disrupts the expression level or distribution pattern of plectin and CD151 within cerebral vessels.

Materials and Methods

Animals

The studies described have been reviewed and approved by the Scripps Research Institute Institutional Animal Care and Use Committee. The generation of $\beta 4$ -EC-KO transgenic mice has been previously described.^[12] Briefly, these mice were generated by breeding Tie2-Cre and $\beta 4$ integrin^{fllox/flox} ($\beta 4$ integrin^{f/f}) strains of mice that have been described previously.^[19,20] Both strains were backcrossed >10 times onto the C57BL/6 background and maintained under specific pathogen-free conditions in the closed breeding colony of the Scripps Research Institute. Breeding of the $\beta 4$ integrin^{f/f} strain with the Tie2-Cre strain generated mice that lacked expression of the $\beta 4$ integrin in endothelial cells ($\beta 4$ -EC-KO), as well as mice that were Tie2-Cre negative, having two copies of the $\beta 4$ integrin gene ($\beta 4^{+/+}$), which were used as controls. Genotyping was performed using previously described protocols.^[19,20]

Chronic Hypoxia Model

$\beta 4$ -EC-KO mice or littermate controls ($\beta 4^{\text{fllox/wt}}$), 8–10 weeks of age, were housed 4 to a cage and placed into a hypoxic chamber (BioSpherix, Redfield, NY, USA) maintained at 8% oxygen for periods up to 14 days. Littermate controls of each strain were also kept in the same room under similar conditions, except that they were kept at normal oxygen

levels (normoxia) for the duration of the experiment. Every few days, the chamber was opened for cage cleaning and food and water replacement as needed.

Immunohistochemistry and antibodies

Immunohistochemistry was performed as described previously^[21] on 10 μm frozen sections of cold phosphate-buffered saline perfused brains taken from mice subject to normoxia (control) or hypoxia for 4, 7, and 14 days. Each slide contained mouse brains representing the four different time-points of hypoxia, to ensure consistent antibody incubation times across different time-points. The following rat monoclonal antibodies were obtained from BD Pharmingen (La Jolla, CA, USA): anti-CD31 (clone MEC13.3), anti- $\beta 4$ integrin (clone 346-11A), and anti-CD151 (clone 455807). Other primary antibodies used included mouse anti- α -SMA-Cy3 conjugate (Sigma, clone 1A4), hamster anti-CD31 (clone 2H8, Abcam, Cambridge, MA, USA), rabbit anti-CD151 (Creative Diagnostics, Shirley, NY, USA), and guinea pig polyclonal anti-plectin (Progen, Heidelberg, Germany). Secondary antibodies used included anti-rat-Cy3, anti-rabbit-Cy3, and anti-guinea pig-Cy3 (Jackson ImmunoResearch, West Grove, PA, USA), anti-Armenian Hamster-Dy-Light 594 (BioLegend, San Diego, CA, USA), and anti-rat Alexa Fluor 488 and anti-guinea pig Alexa Fluor 488 (Invitrogen Corporation, Carlsbad, CA, USA).

Image analysis and quantification

Images were taken using a $\times 20$ objective on a Zeiss Imager M1.m. Analysis was performed in the frontal lobe and medulla oblongata regions of the brain. For each antigen, three images were taken per region at $\times 20$ magnification and the mean was calculated for each subject. All data analyses were performed using Perkin Elmer Velocity software (Waltham, MA). Each experiment was performed with three different animals per condition, and the results were expressed as the mean $\pm$ standard error of the mean. Statistical significance was assessed using the Student's *t*-test, in which $P < 0.05$ was defined as statistically significant.

Results

$\beta 4$ integrin colocalizes with the hemidesmosome proteins plectin and CD151 in cerebral vessels

In a recent study, we demonstrated that in the CNS, $\beta 4$ integrin expression is largely restricted to α -SMA-positive arterioles.^[12] To determine whether the HD proteins plectin and CD151 show a similar expression pattern to $\beta 4$ integrin on cerebral vessels, we performed dual immunofluorescence (dual-IF) on frozen brain sections. As shown in Figure 1 (top row), plectin/ $\beta 4$ integrin dual-IF revealed tight colocalization of these two proteins, with both expressed at the highest level in medium-sized cerebral vessels with a diameter range between 10 and 25 μm . Plectin/CD31 dual-IF confirmed the vascular source of plectin (middle row). Furthermore, CD151/CD31 dual-IF showed that CD151 has a similar expression pattern to $\beta 4$ integrin and plectin, also being expressed at highest levels by medium-sized cerebral blood vessels (bottom row).

In the hypoxic central nervous system, remodeling blood vessels show upregulation of plectin and CD151 in a $\beta 4$ integrin-independent manner

To investigate how the expression pattern of plectin and CD151 is altered during vascular remodeling, we examined

Figure 1: Colocalization of $\beta 4$ integrin with plectin and CD151 on cerebral blood vessels. Dual-immunofluorescence was performed on frozen sections of the frontal lobe from adult mice using antibodies specific for $\beta 4$ integrin (AlexaFluor-488, green), plectin (Cy3, red), CD151 (Cy3, red), or the endothelial marker CD31 (AlexaFluor-488, green). Scale bar = 100 μ m. Note that plectin expression strongly colocalized with $\beta 4$ integrin and that CD151 was also expressed at high levels by the same medium-sized cerebral blood vessels (diameter range 10–25 μ m)

this process in mice exposed to chronic mild hypoxia (8% O_2) for different time periods (4, 7, and 14 days). Under these conditions, a marked angiogenic remodeling response occurs in all areas of the brain, resulting in approximately 50% increased vessel density after 14 days hypoxia.^[22,23] As shown in Figure 2a, plectin/ $\beta 4$ integrin dual-IF revealed that at all time-points examined, $\beta 4$ integrin-positive vessels were always plectin-positive. Interestingly, in the normoxic brain, plectin expression was detected only on $\beta 4$ integrin-positive vessels. However, in the hypoxic brain, a large number of small diameter (<8 μ m) $\beta 4$ integrin-negative vessels also showed plectin expression. Quantification of two different regions of the brain (frontal lobe and medulla oblongata) revealed that the number of plectin-positive vessels per field of view (FOV) was strongly increased during hypoxic-induced vascular remodeling, with the maximal level attained after 7 days hypoxia [Figure 2c]. Compared with normoxic conditions, 7 days hypoxia increased the number of plectin-positive vessels per FOV from 9.7 ± 2.1 to 51.3 ± 10.7 in the frontal lobe ($P < 0.01$) and from 10.1 ± 1.3 to 56.0 ± 6.8 in the medulla ($P < 0.01$). After 14 days hypoxia, by which time vessel remodeling is largely complete,^[22] plectin expression on the smaller vessels had declined to baseline and the expression pattern closely resembled that of the normoxic brain. In addition, hypoxia also induced similar changes in the vascular expression pattern of CD151 [Figure 2b]. Quantification revealed that the number of CD151-positive vessels per FOV was strongly increased during hypoxic-induced vascular remodeling, with the maximal level attained after 7 days hypoxia [Figure 2c and d]. Compared with normoxic conditions, 7 days hypoxia increased the number of CD151-positive vessels per FOV from 9.0 ± 3.0 to 53.7 ± 11.7

in the frontal lobe ($P < 0.01$) and from 8.7 ± 2.2 to 55.6 ± 8.7 in the medulla ($P < 0.01$). This demonstrates that remodeling small caliber vessels show transient upregulation of plectin and CD151 in the hypoxic CNS, but this expression occurs independently of $\beta 4$ integrin expression.

Absence of endothelial $\beta 4$ integrin has no impact on the expression level or distribution pattern of plectin and CD151 within stable or remodeling cerebral vessels

As studies in epithelial tissues have demonstrated an essential role for $\beta 4$ integrin in organizing the localization of plectin and CD151 into HDs,^[6,18] we next examined whether $\beta 4$ integrin plays a similar role in cerebral vessels. To study this, we employed transgenic mice in which $\beta 4$ integrin had been specifically deleted in endothelial cells ($\beta 4$ -EC-KO) as previously described.^[12] If $\beta 4$ integrin is required for organization of HD proteins, it predicts that lack of endothelial $\beta 4$ integrin will lead to disrupted expression of plectin and CD151 in the blood vessels of these transgenic mice. As we cannot visualize $\beta 4$ integrin in $\beta 4$ -EC-KO mice, instead, based on the extremely tight colocalization (1:1) of $\beta 4$ integrin and α -SMA,^[12] we used α -SMA to identify the vessels (arterioles) that normally express $\beta 4$ integrin. As expected, dual-IF with plectin/ α -SMA [Figure 3a] and CD151/ α -SMA [Figure 4a] in the normoxic CNS of wild-type mice revealed very tight colocalization between α -SMA and plectin or CD151. This analysis also confirmed upregulation of plectin and CD151 by small angiogenic vessels in the 4th and 7th day hypoxic brains. However, contrary to our expectation, the expression pattern of plectin [Figure 3b] and CD151 [Figure 4b] on the cerebral blood vessels in $\beta 4$ -EC-KO mice was not appreciably

Figure 2: Increased expression of plectin and CD151 on remodeling blood vessels in the hypoxic central nervous system. Frozen sections of frontal lobe taken from adult mice exposed to normoxia or 4, 7, or 14 days mild hypoxia (8% O₂) were stained with antibodies specific for plectin (Cy3, red) and β4 integrin (AlexaFluor-488, green) (a), or antibodies specific for CD151 (Cy3, red) and β4 integrin (AlexaFluor-488, green) (b). Scale bar = 100 μm. Note that in the normoxic brain, plectin and CD151 strongly colocalize with β4 integrin. However, after 4 and 7 days of mild hypoxia, while all β4 integrin-positive vessels still expressed plectin and CD151, a large number of small caliber (<8 μm diameter) β4 integrin-negative vessels now expressed plectin and CD151. Quantification of the influence of mild hypoxia on vascular plectin (c) and CD151 (d) expression in the frontal lobe and medulla oblongata. Experiments were performed with three different animals, and the results were expressed as the mean ± standard error of the mean of the number of vessels positive for each antigen per field of view. Note that hypoxia promoted a transient increase in vascular plectin and CD151 expression in both areas of the brain, which peaked after 7 days hypoxia, *P < 0.01

different from wild-type controls. In other words, in β4-EC-KO mice, plectin and CD151 expression maintained tight colocalization with α-SMA on medium-sized vessels in

the normoxic CNS, and exposure to mild hypoxia resulted in elevated expression of plectin and CD151 on small vessels [quantified in Figures 3c and 4c]. This demonstrates

Figure 3: Absence of endothelial $\beta 4$ integrin had no impact on the expression or distribution pattern of plectin within cerebral vessels. Frozen sections of frontal lobe taken from wild-type (a) or $\beta 4$ -EC-KO mice (b) that had been exposed to normoxia or 4, 7, or 14 days mild hypoxia (8% O_2) were stained with antibodies specific for plectin (AlexaFluor-488, green) or α -SMA (Cy3, red). Scale bar = 100 μ m. Note that plectin and α -SMA strongly colocalized in the normoxic brain and that after 4 and 7 days of mild hypoxia, many α -SMA-negative small vessels strongly upregulated plectin expression. However, surprisingly, the expression pattern of plectin in the brain of $\beta 4$ -EC-KO mice (b) was no different from wild-type controls (a). (c) Comparison of vascular plectin upregulation in wild-type and $\beta 4$ -EC-KO mice exposed to mild hypoxia. Expression was evaluated in two areas of the brain (frontal lobe and medulla oblongata) with three different animals per strain, and the results were expressed as the mean $\pm$ standard error of the mean of the number of plectin-positive vessels per field of view. Note that hypoxia promoted a transient increase in plectin expression in both strains of mice, with no detectable differences between the two strains

Figure 4: Absence of endothelial $\beta 4$ integrin had no impact on the expression or distribution pattern of CD151 within cerebral vessels. Frozen sections of frontal lobe taken from wild-type (a) or $\beta 4$ -EC-KO mice (b) that had been exposed to normoxia or 4, 7, or 14 days mild hypoxia (8% O_2) were stained with antibodies specific for CD151 (AlexaFluor-488, green) or α -SMA (Cy3, red). Scale bar = 100 μ m. Note that CD151 and α -SMA strongly colocalized in the normoxic brain and that after 4 and 7 days of mild hypoxia, many α -SMA-negative small vessels strongly upregulated CD151 expression. However, surprisingly, the expression pattern of CD151 in the brain of $\beta 4$ -EC-KO mice (b) was no different from wild-type controls (a). (c) Comparison of vascular CD151 upregulation in wild-type and $\beta 4$ -EC-KO mice exposed to mild hypoxia. Expression was evaluated in two areas of the brain (frontal lobe and medulla oblongata) using three different animals per strain, and the results were expressed as the mean $\pm$ standard error of the mean of the number of CD151-positive vessels per field of view. Note that hypoxia promoted a transient increase in vascular CD151 expression in both strains of mice, with no detectable differences between the two strains

that the absence of endothelial $\beta 4$ integrin had no impact on the cellular distribution pattern of plectin or CD151, either on stable or on remodeling cerebral blood vessels.

Discussion

In epithelial tissues, $\beta 4$ integrin plays an important role, organizing a number of structural proteins into specialized adhesive structures called HDs.^[1,2] The importance of this is illustrated by the finding that the absence of $\beta 4$ integrin leads to the loss of organization of specific HD proteins, resulting in severe skin blistering and a perinatal lethal phenotype.^[6,18] In the CNS, $\beta 4$ integrin is most prominently expressed by endothelial cells within arterioles though, unlike the skin, lack of $\beta 4$ integrin does not result in a major structural phenotype in blood vessels.^[12] In light of this difference, the aim of this study was to determine whether $\beta 4$ integrin plays a similar role in the CNS, to organize the localization of the HD proteins, plectin and CD151. Our main findings were as follows: (i) in the normal adult CNS, plectin and CD151 strongly colocalize with $\beta 4$ integrin and are expressed predominantly in arterioles, (ii) in the chronic hypoxic model, where cerebral vessels undergo remodeling, plectin and CD151 are largely upregulated by all cerebral vessels, including $\beta 4$ integrin-negative small diameter vessels, and (iii) the absence of endothelial $\beta 4$ integrin (in $\beta 4$ -EC-KO transgenic mice) had no impact on the expression level or distribution pattern of plectin and CD151 within stable or remodeling cerebral vessels. These results demonstrate that the HD proteins plectin and CD151 are closely associated with $\beta 4$ integrin on arterioles in normal brain and are strongly upregulated on remodeling blood vessels. However, unlike its role in the epidermis, $\beta 4$ integrin is not essential for localization or regulation of expression of plectin and CD151 in cerebral vessels.

Distribution of plectin and CD151 in the central nervous system

Our results in the normal brain show a very tight colocalization between $\beta 4$ integrin and the HD proteins plectin and CD151 within the cerebral vessels. All three proteins were expressed at highest levels in medium-sized cerebral vessels (diameter between 10 and 25 μ m). These findings are largely consistent with previous findings. Errante *et al.* showed that plectin was expressed both on a subset of astrocytes that run along blood vessels and on some endothelial cells and suggested a regulatory role in blood–brain barrier stability.^[16] This was confirmed by subsequent studies, which also showed that plectin expression levels in the white matter were generally higher than in gray matter and that expression was increased on reactive glia.^[14,17,24] In the current study, we unexpectedly found that in the chronic hypoxic model of vascular remodeling, plectin and CD151 were upregulated by all cerebral vessels in a $\beta 4$ integrin-independent manner. Peak expression levels of plectin and CD151 were observed after 4–7 days exposure to chronic mild hypoxia, with a similar time-course to a number of other angiogenic-related proteins, including fibronectin and $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins.^[23,25] This uncoupling of plectin/CD151 and $\beta 4$ integrin expression demonstrates that $\beta 4$ integrin is not essential for expression or localization of plectin and CD151 in cerebral vessels.

Potential roles for CD151 and plectin in the cerebral vessels

CD151 is a member of the tetraspanin family and plays an important role assembling $\beta 1$ class integrins into membrane complexes.^[26,27] CD151 itself has been shown to have an important angiogenic influence. While CD151 null mice show normal vascular development and are viable and fertile, pathological angiogenesis in adult CD151 null mice is perturbed, suggesting a key role for CD151 in this process.^[28] Our findings that CD151 is strongly upregulated on remodeling cerebral blood vessels in the adult suggest that this protein may also influence cerebral angiogenesis. In future experiments, it will be interesting to evaluate hypoxic-induced vascular remodeling response in CD151 null mice. Plectin is an important protein that links adhesion receptors, such as integrins, with intracellular cytoskeletal intermediate proteins, such as keratin in keratinocytes, glial fibrillary acidic protein in astrocytes, and desmin in endothelial cells.^[29] Plectin null mice exhibit a perinatal lethal phenotype, due to defective epidermal integrity, and also exhibit skeletal muscle myopathy and defects in intercalated discs in cardiac muscle.^[30] Specific roles for plectin in endothelial cells have yet to be evaluated.

$\beta 4$ integrin is not essential for localization or regulation of expression of plectin and CD151 within cerebral vessels

Our findings in the chronic hypoxia model showed that angiogenic vessels that were $\beta 4$ integrin-negative, upregulated both plectin and CD151, demonstrating that $\beta 4$ integrin is not required for expression and localization of these HD proteins. This was more thoroughly tested by the use of transgenic mice which lack $\beta 4$ integrin specifically in endothelial cells ($\beta 4$ -EC-KO). Unexpectedly, in $\beta 4$ -EC-KO mice, plectin and CD151 expression levels and pattern were no different from wild-type controls, either in normoxic or hypoxic remodeling conditions, confirming that $\beta 4$ integrin is not required for this process. This came as a surprise in light of the strong data from the plethora of studies in skin, where $\beta 4$ integrin has been shown to be indispensable for regulating localization of HD proteins such as plectin and CD151.^[6,18] Hence, what could explain the difference? One possibility is that other integrins may also perform this function and can compensate in the absence of $\beta 4$ integrin. On this note, several studies have described physical interactions between $\beta 1$ integrin and plectin,^[31,32] and others have shown that CD151 is important for organizing different $\beta 1$ integrins.^[26,27] Alternatively, other integrins such as $\alpha v\beta 3$ might play a role in the organization of HD proteins. Interestingly, a recent study showed that plectin also interacts with the $\beta 3$ integrin,^[33] and this takes on even more value in light of the observation that the angiogenic vessels that switch on plectin and CD151 in the hypoxic brain, also coexpress $\alpha v\beta 3$ integrin.^[25]

Conclusions

Our results demonstrate that the HD proteins plectin and CD151 are closely associated with $\beta 4$ integrin on arterioles in the normoxic brain; however, in the hypoxic brain, plectin and CD151 are widely upregulated on remodeling blood vessels in a $\beta 4$ integrin-independent manner. Furthermore, the absence of endothelial $\beta 4$ integrin (in $\beta 4$ -EC-KO transgenic mice) had no impact on the expression level or distribution pattern of plectin and CD151 within stable or remodeling cerebral vessels. From this, we conclude that $\beta 4$ integrin is not essential for localization

or regulation of expression of plectin and CD151 within cerebral vessels. This suggests that although the main function of $\beta 4$ integrin is to confer stability and integrity in the tissues it is expressed in, the molecular manner in which it achieves this appears to be tissue-specific.

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Conflicts of interest

There are no conflicts of interest.

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