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COMMENTARY

Modeling Williams syndrome with induced pluripotent stem cells

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ABSTRACT

The development of induced pluripotent stem cells (iPSCs) like never before has opened novel opportunity to study diseases in relevant cell types. In our recent study, Williams syndrome (WS), a rare genetic neurodevelopmental disorder, that is caused by hemizygous deletion of 25–28 genes on chromosome 7, is of interest because of its unique cognitive and social profiles. Little is known about haploinsufficiency effect of those deleted genes on molecular and cellular phenotypes at the neural level due to the lack of relevant human cellular model. Using the cellular reprogramming approach, we reported that WS iPSC-derived neural progenitor cells (NPCs) has increased apoptosis and therefore increased doubling time, which could be rescued by complementation of frizzled 9, one of the genes typically deleted in WS. Moreover, WS iPSC-derived CTIP2-positive pyramidal neurons exhibit morphologic alterations including longer total dendrites and increasing dendritic spine number. In addition, WS iPSC-derived neurons show an increase in calcium transient frequency and synchronized activity likely due to increased number of dendritic spines and synapses. Our work integrated cross-level data from genetics to behavior of WS individuals and revealed altered cellular phenotypes in WS human NPCs and neurons that could be validated in other model systems such as magnetic resonance imaging (MRI) in live subjects and postmortem brain tissues.

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Williams Syndrome (WS) is a genetic neurodevelopmental disorder where 25–28 genes on chromosome 7 (7q11.23) are hemizygously deleted (~1.5 megabases) as a result of unequal crossing over of homologous chromosome during meiosis.[1] Haploinsufficiency of these genes causes multiple symptoms including supravalvular aortic stenosis (SVAS), visuospatial deficits, mild to moderate mental retardation, infantile hypercalcemia, craniofacial features (elfin-like faces), hypersensitivity to sound, strong language skills and a very unique social behavior.[2,3] Most of WS patients do not live to normal life expectancy. The death is solely the result of SVAS as elastin which is encoded by *ELN*, is insufficiently produced to form elastic fibers lining the aorta. Thinner elastic fibers then are compensated by increase in number of smooth muscle cells, resulting in less flexible thickened aorta.[4] Unlike patients with most neurodevelopmental disorders including autism spectrum disorders (ASD), Rett syndrome, and down syndrome, who have poor social skills,[5-7] WS patients, on the other hand, are hypersocial and attracted to strangers with the over use of expressive language.[8] Although varied in intensity, the hypersociability is still observed across cultures where children with WS always score relatively higher than typically developing (TD) ones.[9]

WS cases were first reported in 1961 by Dr. John Cyprian Phipps Williams, a New Zealander cardiologist and his colleagues.[10] He noticed that 4 patients with SVAS also shared other common phenotypes including facial features and intellectual disabilities. Nowadays, patients are routinely diagnosed by detection of absence of one copy of *ELN* using fluorescent *in situ* hybridization (FISH) test.[11] The majority of diagnosed WS patients (98%) have classical/typical deletion whereas the others have atypical deletion which could be either as large as ~3.5 Mb[12] or as small as 83.6 kb (*ELN* and *LIMK1 deletion*),[13] resulting in variation in phenotypes. Interestingly, WS individuals with smaller deletion exhibit fewer traits compared with classical/typical WS individuals, suggesting a potential link between the missing typical WS

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phenotypes and genes spared in the genome of those atypical WS individuals.

In the past decades, to reveal mechanism underlying WS characteristics and ultimately establish causal links between genes and WS phenotypes, different models have been used. Human peripheral tissue such as lymphoblastoid cells and fibroblasts provide gene expression profiles that suggest possible alteration in molecular mechanism in different target cell types, including neurons and smooth muscle cells.[14] Verification of such data was not possible at that time as no live source of those human target cell types were available. Single-gene knockout mouse models were also generated.[15-18] While most of heterozygous mice for each gene exhibited some relevant phenotypes, one of them (*Fkbp6*) showed no phenotype at all unless both alleles were knocked out,[19] suggesting that symptomatic traits could be the result of either interaction between more than one gene or the physiologic differences between mouse and human. Recently, a mouse model for WS with full hemizygous deletion has been successfully created and recapitulated most of phenotypes in human including total brain volume reduction, increased social interaction, hypersensitivity to sound and mild motor deficits.[20] Unfortunately, visuospatial learning and memory of WS mice were not tested in this study. While promising, one caveat of this mouse model is that expressive language observed in human could not be understandably assessed in mouse, if any. Functional studies of each gene attributing to WS phenotype were summarized in Table 1.

The use of patient-derived iPSCs to study molecular mechanism underlying pathogenesis has overcome those major obstacles the other disease models faced in the past. Upon reprogramming, these iPSCs possess the abilities closely resembling embryonic stem cells (ESCs) i.e. to self-renew and to differentiate into

Table 1. Mouse and human disease model for genes hemizygously deleted in Williams syndrome.

Gene	Phenotypes observed in single-gene mouse model		Phenotypes observed in rare atypical WS
	Heterozygotes	Homozygotes	
NOL1R	N/A	N/A	N/A
TRIM50	N/A	N/A	N/A
FKBP6	No phenotype observed[19]	Infertility in male[19]	N/A
FZD9	*Moderate* increase in apoptosis in developing dentate gyrus and visuospatial learning/memory deficits[49]	*High* increase in apoptosis in developing dentate gyrus and visuospatial learning/memory deficits[49] Depletion of developing B cell[50]	N/A
BAZ1B	*Moderate* craniofacial abnormalities[51]	*High* craniofacial abnormalities and lethality after birth[51]	N/A
BCL7B	N/A	N/A	N/A
TBL2	N/A	N/A	N/A
MLXIPL	N/A	Lipogenesis reduction[52]	N/A
VPS37D	N/A	N/A	N/A
DNAJC30	N/A	N/A	N/A
WBSCR22	N/A	N/A	N/A
STX1A	No learning and memory deficits[53,54]	Impaired long-term potentiation and memory consolidation[53] High embryonic lethality[54]	N/A
ABHD11	N/A	N/A	N/A
CLDN3	N/A	N/A	N/A
CLDN4	N/A	N/A	N/A
WBSCR27	N/A	N/A	N/A
WBSCR28	N/A	N/A	N/A
ELN	Increase in elastic lamellae and smooth muscle in arteries[4]	Embryonic lethality due to arterial obstruction[17]	Supravalvular aortic stenosis[55]
LIMK1	Generated but never characterized[56]	Altered spine morphology, long-term potentiation, and fear responses[56]	Impaired visuospatial constructive cognition[13]
ELF4H	N/A	N/A	N/A
LAT2	N/A	T-cell activation abnormalities[57]	N/A
RFC2	N/A	N/A	N/A
CLIP2	Moderate growth deficiency and hippocampal dysfunction[18]	Moderate growth deficiency and hippocampal dysfunction[18]	Motor and cognitive deficits[58]
GTF2IRD1	No phenotype observed[59] Mild growth retardation and decrease in fear and aggression[60]	Craniofacial abnormalities[59] Mild growth retardation and decrease in fear and aggression[60]	Visuospatial construction[41,61]
WBSCR23	N/A	N/A	N/A
GTF2i	Increase in social interaction with unfamiliar mice[40]	Embryonic lethality[40]	Hypersociability[41] Visuospatial construction[61]

N/A, neither mouse model nor atypical WS patients for particular gene was available

diverse range of cell types of 3 germ layers (endoderm, mesoderm and endoderm). By ectopically expressing 4 transcription factors (Oct4, Sox2, Klf4 and c-Myc) in adult human fibroblasts, the Yamanaka group was able to induce differentiated cells back to their pluripotent state and demonstrated that these human iPSCs were similar to human ESCs in terms of morphology, proliferation, gene expression and ability to differentiate into several cell types of 3 germ layers.[21] Such advancement has opened up new possibilities that could greatly benefit the medical research, including therapeutic applications and disease modeling.[22-26] First, unlike primary patient cells, which are limited in quantity, iPSCs provide infinite storable source for studies requiring large amount of cells such as high-throughput drug screening. Second, human iPSC-derived cells are more physiologically relevant than animal models and available transformed cell lines. Ultimately, iPSCs allows parallel interdisciplinary studies such as behavioral profiling and organ function and activity monitoring, to be performed in the same individual, facilitating the better interpretation of results integrated across all levels.

Because of unique well-defined genetic background of WS and our interdisciplinary collaboration, we reprogrammed the somatic cells collected from participants in cross-level studies and generated different neural cells to explore the phenotypes in NPCs and neurons (Fig. 1). We have described neuronal phenotypes of NPCs and neurons derived from WS patient together with parallel validation in postmortem neurons.[27]

Despite the availability of current protocols for neuronal differentiation, a major concern in disease modeling using iPSC-derived neural cells that could lead to misinterpretation of the findings is the variation in the neuronal populations generated *in vitro* from each batch of differentiation. We have characterized as much as possible our neuronal culture derived in the study by performing 3 independent analyses. First, a C1 Fluidigm single cell analyses for 96 different genes across several cortical iPSC-derived NPCs and neurons in the different typical WS, atypical WS and TD lines revealed that while NPC populations were very homogeneous among the 3 genotypes, there were consistent differences in sub-populations of post-

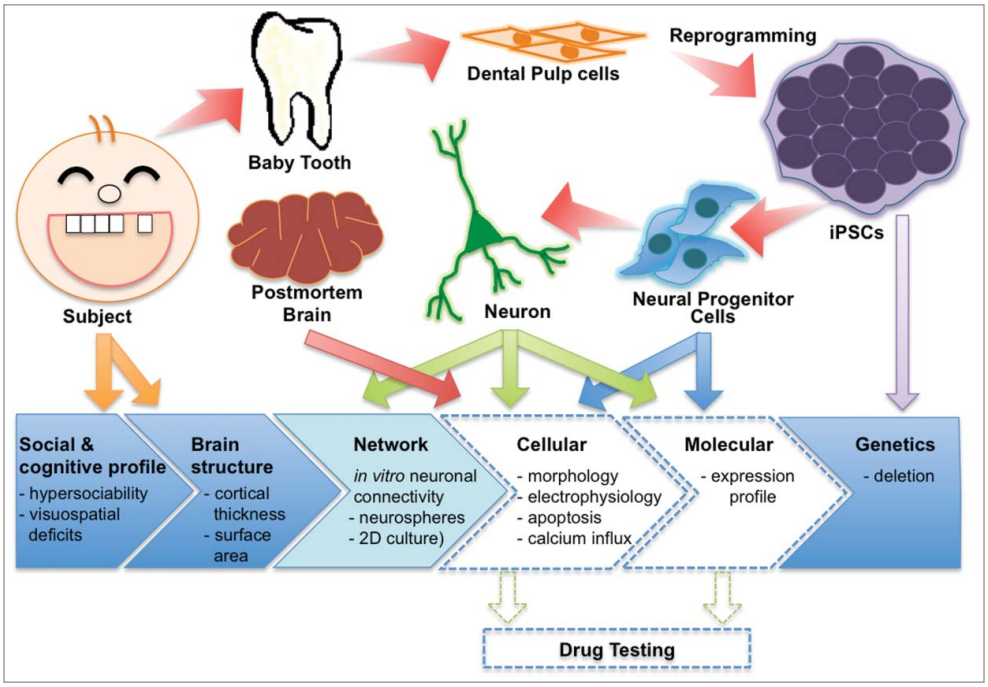

Figure 1. Modeling WS using human iPSCs: Overview. A model to study the neurodevelopmental aspect of WS subjects. We derived iPSCs from dental pulp cells extracted from deciduous tooth of WS individuals whose neurocognitive and social profile was confirmed. Then, WS patient-derived iPSCs were differentiated into neural progenitor cells (NPCs) and neurons for the investigation of specific cellular phenotypes, such as morphology, electrophysiology, apoptosis, calcium influx and expression profile. Concurrently, we analyzed the morphology of postmortem cortical neurons on same subtype we analyzed in the iPSC-derived neuronal culture. Dashed arrows represent future/possible studies.

mitotic neurons. The data clearly showed that the majority of neurons derived from our protocol had a cortical excitatory biased identity (60–80%), some inhibitory neurons (20%) and some glia cells (~30%). Our results also revealed non-significant differences in the number of neurons expressing different cortical layers and neurotransmitters between WS and TD. Second, target gene expression analyses showed the difference in expression level of several genes between WS and TD neurons. Finally, unbiased RNAseq analyses in iPSC-derived sorted neurons confirmed the cortical excitatory nature of our differentiation and pointed out the specific metabolic pathways that are uniquely affected in typical WS compared with TD and atypical WS neurons.

WS cellular phenotypes could be observed early on in precursor cells. WS NPCs have increased doubling time due to an increase in apoptosis, possibly contributing to the decrease in cortical surface area that we observed in WS brains and to the previously reported overall reduction in WS brain volume.[28] Moreover, because of the lack of such NPC phenotype in one atypical WS subject, we were able to restrict this phenomenon to the frizzled 9 (*FZD9*) gene, one of 25–28 genes hemizygously deleted in typical WS, which encodes transmembrane G protein-coupled receptor for Wnt signaling pathway, Using gain and loss of function of *FZD9* itself as well as targeted gene expression analysis of Wnt signaling downstream of *FZD9* and treatment of GSK3 inhibitor, we confirmed the contribution of *FZD9* to NPC survival through canonical Wnt pathway. While we could not exclude the contribution of other genes to other equally relevant phenotypes, our results support that FZD9 is directly responsible for the increased cell death in WS NPCs. As we discussed above, the data open the possibility that FZD9 is responsible for a cortex with less surface area in WS subjects and likely contributes to the disproportional subtypes of neurons in WS.

WS-derived cortical neurons layer V/VI showed several morphometric alterations, including higher total dendritic length and higher number of dendritic protrusions/spines. Rather than the compensatory effect of the surviving NPCs and neurons growing more dendrites and being more active due to the death of WS NPCs, we proved that these phenotypes are intrinsic to WS neurons. We performed a time-lapse of neuronal differentiation among the 3 genotypes by seeding iPSC-derived neurons at the same density and measuring the total dendritic length over time. The result clearly showed a fast growth rate in typical WS neurons compared with TDs and atypical WS. Moreover, we traced TD neurons differentiated from TD NPCs plated at different cellular densities. Within the range of 300–1200 cells/mm^2, we detected no significant differences in the total dendritic length, segment number and spine density. Thus, we conclude that the observed neuronal phenotypes are intrinsic to WS neurons and not due to a compensatory effect.

The morphometric alterations reported for WS cortical neurons go in the opposite direction of neurons derived from autism spectrum disorders, where the social cognition is impaired.[23,25,29,30] Although the findings of the differences in the number of dendritic segments, branching points and trees did not agree between the iPSC model and postmortem tissue, we did observed a similar trend for WS iPSC-derived neurons to have a higher number of dendritic segments and branching points, as observed in WS postmortem brains. It is possible that iPSC-derived neurons lack the dynamics from environmental inputs, and deficiencies in dendritic segments and branching points may occur later in development, resulting in the only partial dendritic changes observed in postmortem specimens. Nonetheless, the fact that we did found similar morphological alternations in both the 2-month-old iPSC-derived neurons in the dish, and in postmortem cortical neurons of WS subjects, not only supports the hypothesis that the iPSC model is capable of recapitulating early neuronal pathology in WS individuals but also suggests that these changes occur early in development and produce life-long effects.

Increases in calcium signaling frequency exhibited in typical WS neurons can be caused by several possibilities including defects in electrical activity generated by changes in intrinsic excitability, in synaptic connectivity, in calcium channels or in calcium pumps. We showed that typical WS neurons could actually form more excitatory synapses compared with TDs. The hyper-connectivity in WS neuronal networks was further validated by electrophysiological recordings using multi-electrode arrays at different time points. Thus, changes in calcium signaling are likely a reflection of the alteration in synaptic activity in WS neurons.

We have shown that, similar to other neurodevelopmental disorders, WS is characterized by pathologies at both macroscopic and neuronal levels. As pathologies in the organization of dendritic trees and

dendritic spines have been long known to represent a feature of mental retardation and developmental disorders,[31-35] functional interpretations of the differences with TD subjects was hindered by the lack of methods to test neuronal activity and to connect the genetics and molecular aspects of diseases with the function and morphology of the neurons. Thus, we have demonstrated that iPSC-derived neurons with morphological changes largely comparable to the ones in the postmortem cortex, displayed differences in doubling time of NPCs and calcium transients in response to activity, both of which may underlie morphological changes observed in individual neurons.[27]

Although the generation of heterogeneous populations of neuron subtypes and astrocytes from iPSCs *in vitro* was achieved in our study, they were 2-dimensional and, therefore, less complex, and much less mature compared with our actual sophisticated brains. Thus, 2D neuronal cultures could possibly recapitulate only cellular defects occurring during early stages of brain development and the findings obtained from such models must be cautiously interpreted. Recently, the development of 3D culture system resulting in brain organoids has been reported,[36-38] offering more closely relevant *in vitro* models for mature brain organization and cellular network. Different differentiation protocols have been developed and fine-tuned to generate organoids with different particular brain region identity,[37,39] potentially allowing study of pathological effects focusing on specific yet complex 3D brain-like structures. When fully established, 3D models would greatly advance disease modeling research especially for complex multigenic neurodevelopmental disorders affecting multiple brain regions like WS.

Future goals

GTF2i and its role in neurons

One of the most interesting genes hemizygously deleted in WS is *GTF2I* (general transcription factor 2i). Because there were several previous studies on this gene compared with others in the WS deleted regions, *GTF2I* is a good candidate for further scientific explorations using reprogrammed cells. Heterozygous knockout GTF2I mice exhibited increased social interaction with 'stranger' mice but no alteration in learning and memory.[40] In atypical WS patient, the subject with hemizygous deletion of all genes except *GTF2I* showed less social interaction with strangers.[41] According to the expression profile of WS fibroblast and lymphoblastoid cells, *GTF2I* was downregulated in WS compared with non-affected individuals.[14] It is ubiquitously expressed in the mouse brain during early development and become restrict to cerebellar Purkinje cells and hippocampal interneurons in adult.[42] Upon ligand stimulation (such as epidermal growth factor), *GTF2I* is tyrosine phosphorylated, resulting in enhancement of transcriptional activity.[43] Besides the role as transcription factor, it has been recently shown that GTF2I might contribute to calcium channel regulation. GTF2I, when activated, competes with TRPC3 (canonical transient receptor potential cation channel 3) for binding to phospholipase C (PLC) via split PH domain, which results in decrease in accumulation of TRPC3 at cell membrane and consequently an inhibition of calcium influx.[44] In WS neurons, haploinsufficiency of GTF2I could potentially lead to an increase in TRPC3 accumulation at cell membrane and therefore an increase in calcium influx. Thus, it is interesting to determine the roles of GTF2I as transcription factor and calcium channel regulator in WS and TD iPSC-derived neurons. The findings will shed light on the cellular basis of differential response to stimulation in WS neurons mediated in part by the activity of GTF2I.

Molecular pathway underlying inverse social behaviors in ASD and WS

ASD, which are mainly characterized by deficits in verbal communication, impaired social interaction, and limited and repetitive interests and behavior, affect about 1 in 68 children in the United States.[45] Although some cases belong to familial forms, majority of them are sporadic.[46,47] Studies of WS could possibly contribute to the understanding in molecular mechanism underlying poor social skills of ASD. Unequal crossing over during meiosis of WS deletion region results in not only hemizygous deletion but also its duplication. The studies reported the cases where the children with this duplication have ASD-like phenotypes including poor social skill, language impairment and developmental delay,[48] suggesting dosage effect of these 25–28 genes on opposite social behaviors. The discovery of molecular pathway alteration in opposite directions in WS and patients with duplication will presumably shed light on hidden players controlling social behavior.

As demonstrated in our previously published studies,[23,25] iPSCs could be used to investigate the functional consequences of *MECP2* mutation (Rett syndrome) and *TRPC6* disruption (non-syndromic autism) on neurons derived from those patients. iPSC-derived neurons from both diseases shared common phenotypes including altered calcium influx, morphological alterations and significant reduction in glutamatergic synapses. These lines of evidence suggest that common pathways, leading to neuronal alterations, may be involved in the etiology of ASDs. In our WS study, same parameters were measured in WS iPSC-derived neurons. Interestingly, while ASD iPSC-derived neurons had decreased total length and fewer branching points compared with control neurons, WS iPSC-derived neurons, on the other hand, had increased total dendritic length and more branching trees. In other words, ASD-derived neurons were less elaborated on their dendritic ramification than WS neurons. We also found that, contrasting to ASD iPSC-derived neurons, calcium transient frequency in WS iPSC-derived neurons was higher compared with control neurons. Using this iPSC system and genome editing technology, we would be able to investigate the impact of haploinsufficiency of each of WS genes in human neurons and explore the hypothesis that different copy number variations can lead to different/opposite biologic effects among WS, idiopathic ASD, ASD-like WS duplication and TD iPSC-derived neurons.

Summary

We revealed WS neuronal phenotypes at cellular levels using iPSCs and differentiation protocols (see summary in Fig. 2). Simple observation of slow growth in typical WS NPCs prompted us to investigate further in NPC proliferation and apoptosis. While proliferation was not altered in all WS NPCs, we found significant increase in apoptosis in only typical WS, not atypical NPCs, suggesting that gene regulating apoptosis in NPCs was spared in atypical WS genome. We showed that the *FZD9* is specifically responsible for an increase in apoptosis in typical WS NPCs as the treatment of sh*FZD9* in TD NPCs and overexpression of *FZD9* in typical WS NPCs mimicked the phenotypes of typical WS and TD NPCs, respectively. We also demonstrated that typical WS iPSC-derived CTIP2+ (layer V/VI) neurons had longer total dendritic length, increased number of dendritic spines and more number of dendritic trees, indicating that they were more elaborate. Most of phenotypes were similarly observed in postmortem cortical layerV/VI neurons except number of dendritic trees. Instead, postmortem neurons had higher number of segments and branching points, which, nevertheless, suggest that they were more elaborate. Moreover, typical WS iPSC-derived neurons also exhibited calcium transient frequency alteration. Thus, the iPSC system is a powerful model for studying complex disease like WS. Further investigation of particular genes could provide more insight

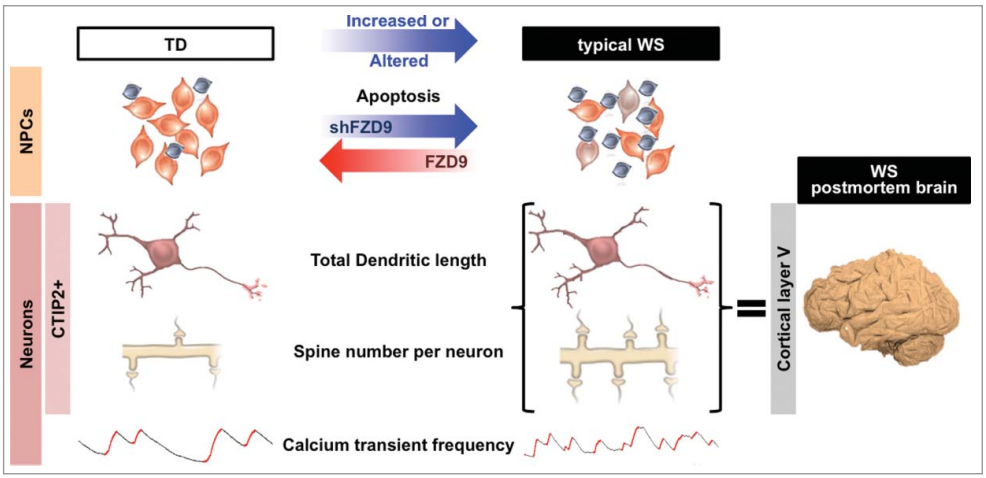

Figure 2. Phenotypic alterations of typical WS iPSC-derived NPCs and neurons. Typical WS NPCs had increased apoptosis compared with TD NPCs. Phenocopy was achieved in TD NPCs treated with shRNA against *FZD9* (shFZD9). Apoptosis defect in typical WS NPCs could be rescued by overexpression of *FZD9*. Morphological analysis of iPSC-derived CTIP2+ cortical neurons (layer V/VI) revealed that WS neurons had increased number of spine per neuron and longer total dendritic length, which were similarly observed in WS cortical layer V/VI neurons in postmortem brain. WS neurons in general exhibited higher calcium transient frequency compared with TDs.

into molecular mechanism underlying etiology of WS and the basis of the human social behavior.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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