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Characterization of a subpopulation of developing cortical interneurons from human iPSCs within serum-free embryoid bodies

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1New York Stem Cell Foundation Laboratory, New York, New York; 2Department of Pathology and Cell Biology and Taub Institute for Research on Alzheimer’s Disease and the Aging Brain, Columbia University, New York, New York; 3Department of Biological Science, Hunter College, New York, New York; 4Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhonpathom, Thailand; and 5The Hussman Institute for Autism, Baltimore, Maryland

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Nestor MW, Jacob S, Sun B, Prè D, Sproul AA, Hong SI, Woodard C, Zimmer M, Chinchalongporn V, Arancio O, Noggle SA. Characterization of a subpopulation of developing cortical interneurons from human iPSCs within serum-free embryoid bodies. Am J Physiol Cell Physiol 308: C209–C219, 2015. First published November 12, 2014; doi:10.1152/ajpcell.00263.2014.—Production and isolation of forebrain interneuron progenitors are essential for understanding cortical development and developing cell-based therapies for developmental and neurodegenerative disorders. We demonstrate production of a population of putative calretinin-positive bipolar interneurons that express markers consistent with caudal ganglionic eminence identities. Using serum-free embryoid bodies (SFEBS) generated from human inducible pluripotent stem cells (iPSCs), we demonstrate that these interneuron progenitors exhibit morphological, immunocytochemical, and electrophysiological hallmarks of developing cortical interneurons. Finally, we develop a fluorescence-activated cell-sorting strategy to isolate interneuron progenitors from SFEBs to allow development of a purified population of these cells. Identification of this critical neuronal cell type within iPSC-derived SFEBS is an important and novel step in describing cortical development in this iPSC preparation.

interneuron; inducible pluripotent stem cell; cortex; calretinin; neuron progenitor

The balance of excitatory and inhibitory inputs underlies efficient information transfer in the central nervous system (51). In the cerebral cortex, circuit dynamics are in part regulated by GABAergic interneurons, which make up the bulk of local synaptic contacts (5). Aberrant interneuron activity has been implicated in the etiology of a number of disorders, including Alzheimer’s disease, autism spectrum condition, and epilepsy (34, 52). The development of interneuron progenitors into mature interneurons is a highly regulated process that originates in the medial, lateral, and caudal ganglionic eminences (MGE, LGE, and CGE, respectively) of the subpallium. In the mouse, 50–60% of all interneurons originate in the MGE and 30–40% originate in the CGE (2, 49). Whereas interneurons originating from the MGE and CGE are primarily cortical, LGE interneurons typically are striatal (13, 43, 48). MGE- and CGE-born pools of interneuron progenitors migrate to the neocortex and other forebrain targets and mature into highly heterogeneous populations of GABAergic interneurons. Classification of these populations has been difficult, but triangulation studies using expression of Ca2+-binding proteins, such as calretinin (CalR) and calbindin, as well as morphological and electrophysiological phenotypes, have yielded general subtypes in mature cells (44).

Using Nkx2.1-mutant mice, Pleasure et al. (37) demonstrated that expression of the transcription factor Nkx2.1 is required for a majority of the MGE-originating somatostatin (SST)-, neuropeptide Y (NPY)-, parvalbumin (PV)-, and calbindin-expressing interneurons in the prenatal cortex (37). Of this population, most MGE-originating interneurons express PV (65%), which is driven in part by Dlx5, Dlx6, Lhx6, and Sox6 (49, 51). However, Dlx5 is also expressed in the subventricular zone of the CGE and aids in regulating migration of CGE-derived progenitors to the cortex in mice (47). Lhx6 is particularly important in identifying MGE-originating PV-expressing interneurons. Interneurons from Lhx6 mutants do not express PV or SST, nor do they migrate to the proper areas of the cortex (17).

While MGE-derived interneuron progenitors have been well characterized using animal iPSC-derived (41) and hiPSC-derived (36) models, the characterization of CGE-derived interneurons has not been appreciated, in part because there are no clear anatomic divisions between the CGE/LGE and the MGE. Additionally, some MGE-migrating progenitors may migrate through the CGE, and some cells have characteristics of both MGE- and CGE-originating neurons (16). Nonetheless, expression of the transcription factors CoupTFI and CoupTFII, as well as Dlx1/2 and Dlx5, has been shown to enhance the migration and expression of CGE-originating cortical interneu-
rons (16, 24). These neurons typically express CalR and have a bipolar morphology (20). A small proportion of CGE-derived interneurons also express VIP and reelin (31). Interneurons with developmental origins in the CGE have a heterogeneous transcriptional lineage but can be characterized in part by morphology and expression of CalR. In vivo fate-mapping experiments demonstrate that CalR+ cells rarely derive from the MGE (1, 48, 45). Because cortical development and function are dependent on a diversity of cortical interneuron subtypes expressing CalR, PV, and SST and these cells seem to originate from distinct, yet somewhat overlapping, regions, it is important to be able to derive them using iPSCs for study in disease models.

Here, we demonstrate our ability to produce a population of putative CalR+ bipolar interneuron progenitors with a transcriptional history consistent with cells that have CGE-like characteristics. Using serum-free embryoid bodies (SEFBs) derived from hiPSCs, we demonstrate that these interneuron progenitors exhibit morphological, immunocytochemical, and electrophysiological hallmarks of cortical interneurons. Additionally, these cells comprise ~30% of the total population of neurons in our SEFB system, consistent with their representation in the cortex in other in vivo and in vitro studies (18, 47). The identification of this group of cells within SEFBs consists of iPSCs is an important and novel step in directing cortical development in this system and refining tools for disease modeling.

METHODS

iPSC culture and neuronal differentiation. iPSC cell lines and culture and differentiation methods are described elsewhere (35, 38, 42). Briefly, undifferentiated hiPSCs (from fibroblasts obtained from the Coriell Institute, Camden, NJ) were maintained on γ-radiated mouse embryonic feeders (GlobalStem, Gaithersburg, MD) in KODMEM with 20% knockout (KO) serum replacement (Invitrogen, Grand Island, NY) containing penicillin-streptomycin (1:100 dilution; all from Life Technologies).

For neuronal differentiation, iPSC colonies were manually cleaned at day 24 with addition of Dickkopf-1 (Dkk-1). After 14 days in 96-well plates, SEFBs are pipetted onto mesh inserts (MIs) in 6-well plates. After 30 days, whole SEFBs are harvested for use in experiments or further subjected to trypsin-based dissociation and replating onto 15-mm glass coverslips coated with polyornithine-laminin (1:6) and allowed to recover for 15 days before processing. QPCR = quantitative PCR.

Table 1. Antibodies used in the study

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Fig. 1. Serum-free embryoid body (SEFB) differentiation and dissociation protocol for interneuron progenitors: outline of differentiation protocol. Inducible pluripotent stem cells (iPSCs) are subjected to dual-SMAD inhibition using an aggregation method from embryoid bodies that are driven toward a frontal fate with addition of Dickkopf-1 (Dkk-1). After 14 days in 96-well plates, SEFBs are pipetted onto mesh inserts (MIs) in 6-well plates. After 30 days, whole SEFBs are harvested for use in experiments or further subjected to trypsin-based dissociation and replating onto 15-mm glass coverslips coated with polyornithine-laminin (1:6) and allowed to recover for 15 days before processing. QPCR = quantitative PCR.
Table 2. Forward and reverse primer sequences used to confirm interneuron targets

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RESULTS

Although our protocol was not specifically designed to produce interneurons, during differentiation of neocortical-like SFEBs on MIs (35) (Fig. 1), a consistent population of CalR+ cells was observed throughout the SFEB and also in and around the cortical rosettes that make up the SFEB (Fig. 2, A and B). Analysis of four different cell lines of MI-grown SFEBs revealed that these lines contained an average of 33.8 ± 3.7% CalR+ cells (n = 5 SFEBs/line), identified as neurons by coexpression with the neuronal markers Tuj1 and DRAQ5 (Fig. 2C).

Additionally, 28% of CalR+ cells in our SFEBs coexpressed Dlx5, a homeobox gene expressed in developing and mature cortical interneurons originating in the MGE and CGE (47) (Fig. 2D). Morphologically, these cells had bipolar dendrites and were postmitotic, similar to previous observations in developing interneurons (8, 51) (Fig. 2E). CalR+ neurons did not coexpress Prox-1 or Tag-1, suggesting that they were interneurons, and not granule cells or pioneer neurons, respectively (Fig. 3, A and B) (6). Because interneuron development and production are critical to understanding the progression of neurodegenerative and neurodevelopmental disease, we characterized this population in the context of whole SFEBs grown on MIs.

To further characterize the CalR+ neurons, we probed the developmental identity of the cells. Interneurons that have an origin in the MGE typically express Nkx2.1 (27, 36). Therefore, SFEBs were made using a Nkx2.1-GFP human iPS line and stained for CalR. Coexpression of Nkx2.1 and CalR would suggest interneurons with a ventral forebrain identity encompassing the preoptic area, septum, diencephalic hypothalamus, and telencephalic MGE (36). Although expression of Nkx2.1 was observed in the SFEBs, there was no significant coexpression of Nkx2.1 and CalR, suggesting that these cells did not have an MGE identity (Fig. 3C). Additionally, staining for Lhx-6 (a marker for MGE-derived interneurons) was also negative in the SFEBs (Fig. 3D). The sporadic expression of Nkx2.1 in these SFEBs can be partially attributed to the use of Dkk-1 in the differentiation protocol, which can enhance MGE fates (23). Taken together, the absence of coexpression of both of these markers in CalR+ cells suggests that the developmental origin of the CalR+ cells in the SFEBs shares some characteristics of a CGE fate.

It has been demonstrated that the transcription factor CoupTFII, the gene that regulates the expression of CoupTFII, is expressed by interneurons migrating tangentially.

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Thus, MI-grown whole SFEBs were fixed and stained for Map2 and CoupTFII to determine the overall content of CoupTFII neurons in the whole SFEB (Fig. 4A). CoupTFII was expressed in the SFEBs (37.1% of neurons in whole SFEBs, n = 4 SFEBs), suggesting that many of the interneurons in this preparation had CGE-like identities. Of these cells, 11.9% coexpressed CoupTFII and CalR and 7.4% coexpressed CoupTFII and GABA (Fig. 4B). Finally, PCR was used to screen the whole SFEB for expression of various interneuron markers, including Gad67, SST, CoupTFII, CalR, reelin, and PV (Fig. 4C). In agreement with the immunocytochemical data, whole SFEBs expressed SST, CoupTFII, and CalR, similar to previous observations for which iPSC protocols were used at similar time points (29). Finally, PCR of whole SFEB lysates showed expression of the transcript Elmo1, specifically expressed in CalR+ cortical interneurons.
Fig. 3. Whole SFEBs grown using the MI protocol are not enriched for medial ganglionic eminence (MGE)-derived progenitors. SFEBs \((n = 5)\) do not express \(\text{Tag-1} (A)\) or \(\text{Prox-1} (B)\). C: SFEBs grown using an \(\text{Nkx2.1-GFP reporter line on MIs do not contain a significant number of cells that coexpress CalR (red) and Nkx2.1 (green; arrows).} \) D: SFEBs \((n = 5)\) do not coexpress \(\text{Lhx-6 (green) and CalR (red), suggesting that CalR cells are not consistent with MGE-born interneurons. Scale bars } \equiv 20 \mu\text{m.} \)

Fig. 4. Whole SFEBs express a subpopulation of cells with markers consistent with developing interneurons from the caudal ganglionic eminence (CGE). A: \(\text{CoupTFII (red) and CalR (green) staining in SFEBs } \equiv 5\) shows that MI-grown SFEBs are enriched for cells expressing \(\text{CoupTFII. Scale bar } \equiv 10 \mu\text{m.} \) B: quantitative representation of \(\text{CoupTFII-coexpressing populations in SFEBs.} \) C: PCR of whole SFEB shows expression of interneuron markers \(\text{SST, CoupTFII, CalR, and Gad67. CNTN4, contactin 4; SST, somatostatin; RELN, reelin; PV, parvalbumin.} \) D: PCR of whole SFEB shows expression of \(\text{Elmo1, but not Hox10, indicating that interneurons from this protocol do not originate from the spinal cord.} \)
migrating from the CGE (18), and the absence of the spinal cord marker Hox10 (Fig. 4D). These results suggest that SFEBs grown on MIs using our differentiation protocol can produce a subpopulation of CalR<sup>+</sup> interneurons, a large percentage of which demonstrate a transcriptional profile similar to that observed in CGE-derived CalR<sup>+</sup> interneurons.

To further study these CalR<sup>+</sup> neurons, SFEBs were subjected to trypsin dissociation and trituration to break up the tissue. The dissociated cells were replated onto poly-o-laminin-coated coverslips (Fig. 1). This manipulation of the preparation allowed us to perform electrophysiological recordings and stain the neurons concurrently. This approach is novel, in that it allows the development of these cells in a three-dimensional (3-D) context, after which they can be used with traditional monolayer preparation techniques.

The patch-clamp mode was used to record and analyze passive and active membrane properties of neurons recorded from dissociated SFEBs. During the course of recording, cells were backfilled with Alexa 488 via the patch pipettes to qualitatively compare the single-cell morphology of these cells with that of CalR<sup>+</sup> cells in SFEBs in the earlier experiments. Qualitatively, these cells showed bipolar morphology similar to that of cells in intact SFEBs (Fig. 5A). Although we could not specifically patch-clamp CalR<sup>+</sup> interneurons, evoked action potentials from the cells could be blocked with 1 µM TTX (data not shown), and capacitance (13.4 ± 1.3 pF), input resistance (468.7 ± 117.9 MΩ), and resting membrane potentials (−48.9 ± 1.8 mV) were observed (n = 10). These values were comparable to those for MGE-derived interneurons and also to previously reported data for regular-spiking nonpyra-

![Image](http://ajpcell.physiology.org/)

**Fig. 5.** Electrophysiological properties of interneuron-like cells from dissociated SFEBs. A: single CalR<sup>+</sup> neuron from an intact SFEB (left) and single CalR<sup>+</sup> neuron from a dissociated SFEB (right) backfilled with Alexa 488. Both show a qualitatively similar morphology. A and B: a representative cell patch-clamped in this study. Recordings taken from the neuron backfilled in A show normal action potentials with a non-fast-spiking pattern (single trace at right in B) that resembles a developing interneuron (n = 10 cells). Scale bars = 20 µm. C: a representative miniature inhibitory postsynaptic current recording from a neuron taken from dissociated SFEBs blocked by picrotoxin (n = 11 cells). D: a single GABAergic current evoked through pressure ejection of GABA (0.5 mM) recorded from a neuron within a dissociated SFEB blocked by picrotoxin (n = 10 cells). E: expression of CalR, SST, and Gabra1 in a PCR obtained from a dissociated coverslip obtained from preparation used for recording in C.
midal CalR⁺ interneurons in the monkey prefrontal cortex (29, 36, 54) (Fig. 5B).

To assess the presence of interneurons in dissociated SFEBs, mIPSCs were recorded in the presence of 1 μM TTX, 100 μM D-APV, and 10 μM CNQX added to the bath solution to block spontaneous action potentials and glutamate neurotransmission. We observed that 45% of the recorded neurons (n = 11) showed mIPSCs that could be blocked by PTX (Fig. 5C), with an average frequency of 32.7 ± 17.1 events per minute (n = 5) and an average amplitude of 15.1 ± 0.91 pA (n = 5).

GABA is the typical neurotransmitter for ~99% of the CalR⁺ cells in the mouse neocortex and 75% of the CalR⁺ interneurons in the monkey and human prefrontal cortices (14, 19, 21, 26, 30). Thus, to more specifically characterize the neurons taken from dissociated SFEBs after plating, GABA (0.5 mM) was directly applied using pressure ejection during recording in voltage-clamp mode at a holding potential of ~80 mV. A GABA response (n = 16) that could be blocked by PTX was observed in 50% of the recorded neurons. The average amplitude of the response was ~452 ± 91.6 pA (n = 8). These data suggest that, after dissociation and plating of MI-grown SFEBs, cultures were enriched for neurons that had developing interneuron-like electrophysiological properties.

After recording, the coverslip was lysed and PCR was used to screen for common developing interneuron markers (Fig. 5E). Total lysates revealed strong bands for CoupTFII and Gad67 and weaker expression for CalR. Coverslips were also fixed and stained for interneuron markers observed in whole SFEBS. While we were not able to localize the exact cell from which we recorded, we were able to locate the region within 100 μm of the recording electrode after fixation and staining. Staining revealed that 33.9% of the cells expressed CalR and 26.6% expressed GABA (Fig. 6B). We also observed that 9.8% expressed Dlx5 and 11.6% expressed CoupTFII (Fig. 6, A–C). Interestingly, at this early time point, a small percentage (4.8%) also expressed SST, suggesting that these cells express markers consistent with differentiation into more mature interneurons (Fig. 6D). To isolate a population of cells with interneuron-like properties from whole SFEBS, we employed FACS using CD133 and CD184 antibodies, a strategy analogous to a recently published protocol used to isolate mature neurons (53) (Fig. 7A). CD184 (CXCR4) has been described as an upregulated marker in the developing mouse cortex (4), suggesting that it is useful in preferential selection of developing interneurons. CD133, a well-characterized neural stem cell marker, was chosen to isolate a more mature and committed precursor population (39). Dissociated SFEBS were first negatively gated on CD133 to remove multipotent neuronal progenitors and then selected positively or negatively on CD184 to screen for common developing interneuron markers (Fig. 6D). To isolate a population of cells with interneuron-like electrophysiological properties.

DISCUSSION

The ganglionic eminences, including the MGE and the CGE, are responsible for the majority of cortical interneurons in vertebrates (49). Using 3-D iPSC culture protocols based on previous protocols (35), we have described a system that produces a distinct population of CalR⁺ neurons that have interneuron-like characteristics and are in the early stages of development. Additionally, these neurons express markers consistent with a lineage that appears to mimic aspects of CGE-migrating interneurons. We have demonstrated that sim-
ple trypsin-based dissociation and replating can enrich this population, and FACS can isolate a subpopulation of cells that express markers consistent with developing GABAergic interneurons.

The use of 3-D SFEBs in this protocol allows the development of cortical rosettes with anterior-to-forebrain-originating interneuron subtypes. This differentiation protocol results in progression of the expression of cortical markers with a developmental origin and time course similar to that observed in Sasai-style SFEBs (15). The majority of the cells we studied expressed CalR as opposed to the more mature interneuron marker SST (which is responsible for 20% of the cortical interneurons in mice and rats), although we did observe a small percentage of SST-expressing cells at day 30 in our preparation (20, 25). In mice and rats, MGE-derived interneurons rarely express CalR (49). However, CalR was highly expressed in our human iPSC-derived SFEB preparation. Indeed, Wonders and Anderson (49) speculate, on the basis of the work of Rogers (40), that the origins of CalR+ interneurons may be anatomically and temporally different from those of PV and SST+ interneurons. The finding that <5% of cells in the dissociated SFEBs also expressed SST corresponds with a recent report by Nicholas et al. (36). Using an embryoid body protocol generating MGE-originating interneuron populations, they observed that 3% of the interneurons expressed SST at 10 wk postdifferentiation, whereas our cells were 4 wk postdifferentiation. It is possible that the SST expression we observed is attributable to cells with expression profiles that suggest a MGE fate. SST expression is not limited to the MGE, however, as SST-expressing cells have been observed in cells with a CGE origin (33). Additionally, our data showing expression of Elmo1, a transcription factor that is important for the activity-dependent migration of CGE-derived interneurons (18), in whole SFEBs suggest that the majority of SST-expressing cells that we observed are consistent with a CGE fate.
The interpretation of these results relies on the expression of the transcription factor CoupTFII as a readout for CGE lineage. It is difficult to clearly interpret the transcriptional and developmental identities of the CalR+ cells in this study for two reasons: 1) delineation of the CGE is largely based on anatomic studies, and the CGE shares border regions with the LGE and MGE, and these borders are still not clearly defined in the literature (49), and 2) some MGE-derived cells migrate through the CGE to the cortex, and gene expression patterns are similar in the LGE and CGE (16, 28, 33). Nonetheless, it has been demonstrated that expression of CoupTFII is primarily localized to cells that have a transcriptional history consistent with a CGE lineage and is responsible for promoting their migration to the cortex (24). Additionally, the majority of neurons that express CalR and have bipolar morphologies were shown to have a CGE lineage (7, 51). These two criteria describe the cells that we have isolated from SFEBs in this study.

Comparison of cells in dissociated SFEBs with cells in intact SFEBs shows a distinct decrease in the percentage of neurons that express CoupTFII (26% in whole SFEBs vs. 11.6% in dissociated SFEBs), whereas CalR+ cells remain relatively constant. This result may be due in part to the trypsin dissociation of the SFEBs. Trypsin dissociation buffer contains EDTA, which helps deplete extracellular Ca2+ and, thus, promotes dissociation. However, this leads to some apoptosis and changes in cellular morphology (3). It is not understood why sensitivity to trypsin dissociation would be greater in CoupTFII-expressing neurons than in others. It is possible that because CGE neurons are born first and are the most mature, they may be more sensitive to dissociation than neurons born later. Future studies will address the role of the dissociation agent in preserving more of the CoupTFII-expressing population after SFEB dissociation.

In a previous study (35), we did observe some Pax6 staining, suggesting that, along with CGE-like progenitors, there may be immature neurons with characteristics of MGE-like cells in our preparation. However, in a recent study using tissue from human fetal forebrain, Pax6 expression was observed in the ventricular zone of the lateral LGE and the subventricular zone of the CGE, but not in the MGE (32). Because of the close anatomic arrangement of these areas in the developing telencephalon and the ambiguity of their delineation, it is not surprising that some of the SFEB progenitors in previous studies have shown expression of MGE markers, especially as our protocol involves the addition of recombinant Dkk-1, a Wnt inhibitor, which has been shown to enhance MGE fates (23). However, the focus of this study is characterization of the distinct subpopulation of interneurons that were observed to be CalR+ with no MGE marker overlap. In this study, SFEB formation using an Nkx2.1-GFP line resulted in no significant coexpression with CalR+ cells, which is consistent with the idea that the majority of CalR+ cells in our protocol share characteristics with cells that have a CGE-like lineage.

Although CalR+ cells were observed in the whole SFEB, we wanted to isolate these cells to further understand their physiology, as well as to develop a protocol for purifying them for applications such as disease modeling and drug discovery. We developed a novel FACS strategy to try to isolate the interneuron subpopulation within the SFEB. It is unclear why CD133+/CD184− cells seem to enrich for cells with interneuron-like properties (rather than neurons in general), particularly in light of some reports indicating that CD138 (CXCR4) is upregulated in Dlx5+ interneurons in mice (4). Our leading hypothesis is that CD133+/CD184− cells would selectively enrich the earliest-born and most-mature neurons and, thus, might select early-born interneurons in our SFEB preparation. Perhaps a temporal expression of CD184 exists within this context to sort interneuron populations. Future studies will look more carefully at the birthdays of interneurons vs. glutaminergic neurons in the SFEBs to test this possibility and to refine the strategy to more clearly sort for cells that have CGE-like properties.

The observation of CalR+ neurons in the MI-grown SFEBs allows for further understanding of the development of this differentiation protocol and describes the developmental time course of the cortical structures in our system. On the basis of these observations and those of the previous study (35), we are able to develop a 3-D iPSC culture system that more accurately recapitulates cortical development and can be used for more accurate disease modeling and drug screening. By isolating CalR+ progenitors with properties consistent with a CGE lineage and enriching them via trypsin-based dissociation and FACS, this protocol, along with that developed for MGE-derived interneurons from hiPSCs (36), can be used together to mix interneuron subtypes with distinct anatomic and developmental identities. The combination of interneuron subtypes with glutamatergic neurons and astrocytes also described in MI-grown SFEBs provides a powerful tool for using iPSC-derived neurons to model neurodegenerative disorders and understanding normal cortical development.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
M.W.N., S.J., A.A.S., and S.A.N. developed the concept and designed the research; M.W.N., S.J., B.S., D.P., A.A.S., S.I.H., C.W., M.Z., and V.C. performed the experiments; M.W.N., S.J., B.S., D.P., A.A.S., S.I.H., C.W., and M.Z. analyzed the data; M.W.N., S.J., B.S., and A.A.S. interpreted the results of the experiments; M.W.N., S.J., B.S., and A.A.S. prepared the figures; M.W.N. and S.J. drafted the manuscript; M.W.N., S.J., B.S., D.P., A.A.S., M.Z., O.A., and S.A.N. edited and revised the manuscript; M.W.N., S.J., B.S., D.P., A.A.S., O.A., and S.A.N. approved the final version of the manuscript.

REFERENCES


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