A Modified Monomeric Red Fluorescent Protein Reporter for Assessing CRISPR Activity

Camilla Højland Knudsen, Emilía S. Ásgrímsdóttir, Karim Rahimi, Katherine P. Gill, Sas Frandsen, Susanne Hvolbøl Buchholdt, Muwan Chen, Jørgen Kjems, Fabia Febbraro and Mark Denham

Gene editing in human embryonic stem cells (hESCs) has been significantly enhanced by the discovery and development of CRISPR Cas9, a programmable nuclease system that can introduce targeted double-stranded breaks. The system relies on the optimal selection of a sgRNA sequence with low off-targets and high efficiency. We designed an improved monomeric red fluorescent protein reporter, GEmCherry2, for assessing CRISPR Cas9 activity and for optimizing sgRNA. By incorporating an out-of-frame sequence to the N-terminal of the red fluorescent protein mCherry, we created a visual tool for assessing the indel frequency after cutting with CRISPR Cas9. When a sgRNA-Cas9 construct is co-transfected with a corresponding GEmCherry2 construct, single nucleotide indels can move the GEmCherry2 sequence back in-frame and allow quantification and comparison of the efficiency of different sgRNA target sites by measuring red fluorescence. With this GEmCherry2 assay, we compared four target sites in the safe harbor AAVS1 locus and found significant differences in target site activity. We verified the activity using TIDE, which ranked our target sites in a similar order as the GEmCherry2 system. We also identified an AAV short inverted terminal repeat sequence within the Cas9 construct that, upon removal significantly improved transient transfection and expression in hESCs. Moreover, using GEmCherry2, we designed a sgRNA to target SORCS2 in hESCs and successfully introduced indels into the coding sequence of SORCS2.

INTRODUCTION

Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are an invaluable cell source for recapitulating and studying early developmental or disease processes. Furthermore, refined differentiation methods capable of generating patient-specific cell derivatives have the potential to improve cell replacement therapies for numerous diseases, including neurodegenerative diseases such as Parkinson’s disease. In addition to hiPSC technology, recent advancements in gene editing techniques have aided these efforts by increasing the ease at
which genome editing can be performed, for applications including correction or deletion of genes or creation of reporter cell lines for the study of developmental processes.

During the last decade, several gene-editing techniques have been developed, such as Zinc finger nucleases (ZFN) and the transcription activator-like effector nucleases (TALENs) which feature a customizable DNA binding domain, allowing its protein sequence to be modified to target specific genomic sequences. The requirements to custom-modify the protein sequence for each target site limits its broad application. Alternatively, the clustered regularly interspersed palindromic repeats (CRISPR) associated nuclease Cas9 system can be modified to target specific DNA sites; this system is simple to customize as it uses the same protein sequence in each case. The type II CRISPR Cas9 system requires only the modification of a synthetic single guide RNA (sgRNA), which can be programmed to identify a 20 base-pair sequence that is followed by a protospacer-adjacent motif (PAM) sequence -NGG. This sgRNA modification is therefore inexpensive and straightforward in comparison to ZFN and TALENS. Furthermore, this system results in a site-specific double-stranded break (DSB) between the 17th and 18th bases in the target sequence, unlike the indiscriminate cutting employed by the FokI endonuclease used in ZNF and TALENS. The CRISPR system is therefore easily programmed and results in a site-specific DSB, making it a valuable tool for iPSC genome engineering.

Previous reports have shown that the guide RNA architecture and targeting site can both influence Cas9 activity (Sander and Joung, 2014). Several methods for assessing Cas9 activity has been reported, including the use of a surrogate dual fluorescent reporter system, SURVEYOR, RFLP, TIDE, whole genome, or exome sequencing (Kim et al., 2011; Ran et al., 2013b; Brinkman et al., 2014; Cho et al., 2014; Kuhar et al., 2016; Zhou et al., 2016). Although all these systems can determine Cas9 activity, some also capable of assessing off-targets, they are somewhat hampered by either low sensitivity, labor-intensive procedures, or use multiple fluorescent proteins that prevent the assessment of Cas9 co-transfection. Here we describe the development of a modified monomeric red fluorescent protein (RFP) for measuring Cas9 activity, which serves as a rapid tool for evaluating sites with high Cas9 activity. We demonstrate that this technique can be rapidly employed to identify highly efficient sites within the AAVS1 safe harbor locus or in a protein coding sequence. Furthermore, for efficient plasmid transfection of Cas9 sgRNA constructs into hPSCs we increased transient expression by removal of an AA VS1 region was carried out with OneTaq hot start DNA polymerase (NEB) as described by the manufacturer, under a fluorescent microscope at 48 h post-transfection. After imaging, the cells were trypsinised (0.25% trypsin-EDTA) to a 20% confluency of 2 µg of total DNA (no more than 2 µL) and transferred to a Nucleofection chamber (16-well strip). The H9 cells were transfected using parameter CM 130, P3 solution, and plated on Vitronectin coated plates (35 mm, Nunc) in E8 media supplemented with 10 µM ROCK inhibitor. Media was changed to regular E8 media after 1–2 days.

Fluorescence Activated Cell Sorting (FACS) analysis
HEK293T cells were co-transfected with GE mCherry and corresponding CRISPR-Cas9 vectors. The cells were imaged under a fluorescent microscope at 48 h post-transfection. After imaging, the cells were trypsinised (0.25% trypsin-EDTA) to a single cell suspension, washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 20 min at 4°C. The fixed cells were washed and put through a 40 µm cell strainer before being sorted against GFP and mCherry on an LSR Fortessa cell analyser.

Polymerase Chain Reactions (PCRs)
PCR of genomic AAVS1 regions was carried out with OneTaq hot start DNA polymerase (NEB) as described by the manufacturer, with an initial denaturation step of 4 min and an annealing temperature of 63°C. Primers are listed in (Figure S1).
Cloning
GEmCherry constructs were produced using the pmCerulean3-N1 (Addgene: 54730) backbone and cloning in the mCherry gene by PCR amplification from E[βα]C (Addgene: 24312) and N-terminal changes added by primer extension, producing the following vectors: pGEmCherry1, pGEmCherry2, pGEmCherry3, pGEmCherry4. The CBh promoter in pSpCas9(2B)-2A-GFP (PX458) (Addgene: 48138) was swapped for the CAG promoter, which was amplified by PCR from pPB-CAG.OSKM-puDtk (Yusa et al., 2009) producing the final vector pCAG-SpCas9-2A-GFP. The GEmCherry vector was cut with XbaI and BamHI (NEB) to allow insertion of target sites. The oligos (target sequences in Figure S1) were annealed before ligating them into the backbone finally producing GEmCherry2-C2, GEmCherry2-C3, GEmCherry2-C4, GEmCherry2-T2, GEmCherry2-TS1+3, and GEmCherry2-TS2. The Cas9 vector was constructed according to protocol (Ran et al., 2013b), producing pSpCas9-2A-GFP-sgC2, pSpCas9-2A-GFP-sgC3, pSpCas9-2A-GFP-sgC4, pSpCas9-2A-GFP-sgT2, pSpCas9-2A-GFP-sgC2, pSpCas9-2A-GFP-sgSorcs2-G1, pSpCas9-2A-GFP-sgSorcs2-G2, pSpCas9-2A-GFP-sgSorcs2-G3, and LentivCRISPR2-sgSorcs2-G2. Cloning was carried out using electrocompetent Stbl3 or Top10 E. coli cells. The AAV ITR fragment was removed from pCAG-SpCas9-2A-GFP by double digestion with NotI and SbfI to remove a 145 bp fragment including the ITR element. After removing the ITR element, two ssDNA oligos were synthesized (Sigma) and annealed to produce a fragment containing a BamHI site with two sticky ends compatible with the NotI and SbfI and ligated in with T4 DNA ligase (ThermoFisher EL0016) according to the manufacturer instructions. The following vector without an ITR was produced: pCAG-SpCas9-2A-GFP-noITR. All plasmids have been made publicly available through the Addgene non-profit plasmid repository.

Lentiviral Production and Infection
Lentivirus was produced similarly to what was previously described (Denham et al., 2010). HEK293T cells were transfected by Lipofectamine 2000 with plasmids LentiCRISPRv2_SORCS2-G2, envelope plasmid pMD2.G, and packaging vector psPAX2 to generate lentiviral particles. The viral supernatant was concentrated after 48 and 72 h post-transfection and concentrated by ultracentrifugation. Single cells of H9 on Vitronectin coated 35 mm dishes (6,000 cells per dish) were infected with concentrated lentivirus. At 48 h post-infection, Puromycin was added for 5 days (0.125 μg/mL Puromycin; ThermoFisher).

Immunostaining
Immunocytochemistry was performed on cells grown on coverslips (VWR, Ø: 13 mm) and fixed in 4% PFA. Samples were blocked for 1 h at room temperature with 5% donkey serum in PBS-T (PBS with 0.25% triton-X). Primary antibodies were applied in blocking solution at room temperature overnight (GFP, rabbit, abcam: ab290, 1:1000; POU5F1, mouse IgG2b, Santa Cruz: sc-5279, 1:100) and washed off, 3x 10 min, with PBS-T and samples blocked for 10 min before applying secondary antibody in block solution for 1 h at room temperature (AlexaFluor 488 donkey anti-rabbit, 1:1000; AlexaFluor 568 donkey anti-mouse IgG (H + L), 1:1000). The secondary antibodies were washed off 3x 10 min in PBS-T before applying DAPI (1:1000 in PBS) for 10 min at room temperature. Coverslips were washed 3x 5 min with PBS before mounted on microscope slides with PVA DABCO (Sigma Aldrich).

Spectral Imaging
Emission wavelength and intensity of GEmCherry1, 2, and 3 was measured using λ-scan with ZEISS Confocal LSM800 microscope. Intensity was normalized within each sample and then plotted against emission wavelength in PRISM GraphPad 6.

Sequencing
Sanger sequencing was carried out by EurofinsGenomics in ValueRead or Mix2Seq tubes. Sequencing samples were prepared according to manufacturers guidelines but with 300 ng of PCR product per sample. Sequencing primers are described in (Figure S1).

Statistical Analysis
FACS analysis was performed on 50,000 events per replicate using FlowJo software. Statistical analysis, consisting of one-way ANOVAs, adjusting for multiple comparisons with Tukey’s correction, and unpaired two-tailed t-test was done using PRISM GraphPad 6. Mean gray-scale value was found using FIJI ImageJ on images taken with Zeiss Apotome microscope (no apotome function). Quantification of GFP/POU5F1 positive cells was done by measuring pixel area using FIJI ImageJ on images from Olympus IX53 microscope.

TIDE Analysis
TIDE analysis was performed on the AAVS1 locus (PCR primers listed in Figure S1) from cultures sorted for GFP positive cells using the tide.nki.nl tool (version 0.1; Brinkman et al., 2014) run in R. The alignment window was set manually from where the Sanger sequencing chromatogram looked reliable and until 10 bp before the break site (corresponding R function modified to fix alignment window; code is available via osf.io/97ef4). The decomposition window was set at 400 bp, starting 42 bp downstream of the break site. The indel size range was set at 38 bp and the p-value significance cut-off at 0.001.

The reported efficiency of sgRNAs is likely an underestimate due to primer design, which did not allow for comparative analysis of an indel size range larger than 38 bp.

RESULTS
Development of a Monomeric RFP Reporter for Measuring CRISPR Activity
In order to obtain the highest targeting efficiency using CRISPR/Cas9 we first sought to develop a rapid system for evaluating sgRNA efficiency by developing a single fluorescent system to assess Cas9 activity that could be used in conjunction with existing CRISPR Cas9 GFP plasmids, such as the pCas9-GFP. This approach would allow for FACs based or visual assessment of double positive GFP/RFP fluorescence, resulting
Identification of an Optimal CRISPR AAVS1 Site

After successfully engineering a single fluorescent protein, GEmCherry2, which can facilitate an out-of-frame N-terminal fusion for measuring Cas9 activity, we tested whether GEmCherry2 could be used to measure Cas9 activity for a given genomic sequence. We chose the AAVS1 locus (PPPIR12C) as a site, since it has been targeted successfully before with ZFN, TALENS, and CRISPR/Cas9 (Hockemeyer et al., 2009; Dekelver et al., 2010; Mali et al., 2013). Furthermore, this site is a known safe harbor locus, which makes it suitable for future transgene targeting. Firstly, we identified sequences with the lowest off-targets by CRISPOR (Haeussler et al., 2014). From this, we selected three sites designated C2, C3, and C4 (Figure S1) for assessing sgRNA Cas9 activity. These three sites were compared to the T2 site commonly used by others (Hockemeyer et al., 2009). Recognition sequences were cloned into GEmCherry2 and sgRNA sequences into the Cas9-GFP vector. Co-transfection and FACS analysis revealed that significant variation in Cas9 activity was observed between the genomic sequences (Figures 2A,B), confirming that sgRNA architecture and recognition site sequence contributes toward Cas9 activity. Based on GEmCherry2 fluorescence, we determined that sgRNA C4 had the highest efficiency, at 5.504 times that of C3 (p < 0.0001). C2 and T2 were also significantly greater than C3 [C2 (4.803), p < 0.0001; T2 (4.493), p < 0.0001]. No significant difference between C4, C2, and T2 were observed. Based on CRISPOR, C4 contained only two in-exon off-targets, both with four mismatches, whereas the T2 site contained 21 in-exon off-targets each with four mismatches and one in-exon off-target with a three base-pair mismatch (Figure S2). Overall, the off-target assessment and cutting efficiency of C4 makes it a superior choice when targeting the AAVS1 locus with high Cas9 activity and the lowest potential off-targets.

Comparative Efficiency Assessment of GEmCherry2 and TIDE Assay

Having determined that GEmCherry2 can be used to assess sgRNAs and Cas9 activity, we further sought to validate this technique and compare the efficiency determined by GEmCherry2 to Cas9 activity in the genomic locus. Epigenetic modifications can influence the ability of Cas9 to access the genomic DNA and may affect its activity in a genomic context. Therefore, we performed the TIDE assay (Brinkman et al., 2014) on the same CRISPR AAVS1 target sites and calculated the indel frequency as a measure of Cas9 activity. The TIDE assay revealed that, similar to the GEmCherry2 assay, Cas9 activity varied between these sites (Figures 3A,B). Interestingly, the ranking of the various sites was in a similar order to that identified by GEmCherry2, with both methods identifying the AAVS1-C4 sgRNA as the most efficient (Figures 2B, 3A,B). This data reveals that GEmCherry2 can be effectively and rapidly used as a method for determining Cas9 activity.

Gene Editing in Human Embryonic Stem Cells

Finally, we used GEmCherry2 to design a sgRNA to knockout a protein-coding gene in hESCs. We chose to target SORCS2, a proneurotrophin receptor expressed in neurons and we designed three sgRNA against regions in the first coding exon (Glerup et al., 2014). Evaluating these with GEmCherry2, we identified sgRNA SORCS2-G2 as the most efficient sgRNA (Figures 4A,B). To introduce Cas9 and the sgRNA into hESCs, we tested the plasmid pSpCas9(BB)-2A-GFP (Ran et al., 2013b). After transfection, we found that transient expression from the Cas9 constructs [pSpCas9(BB)-2A-GFP (PX458); Addgene: 48138] was almost absent in comparison to other GFP reporter plasmids of similar size (data not shown). We therefore modified the plasmid and exchanged the Cbh promoter for a stronger CAG promoter, resulting in the plasmid pCAG-SpCas9-2A-GFP, which only resulted in
FIGURE 1 | Development of monomeric RFP reporter for measuring CRISPR Cas9 activity. (A) Protein sequence of mCherry, GEmCherry1 with N-terminal modification to allow insertion of 23 bp target site, GEmCherry2 with two ATG→CTG mutations to decrease background fluorescence, GEmCherry3 with three ATG→CTG mutations and GEmCherry4 with two ATG→TTA mutations. (B) Images of HEK293T cells transfected with in-frame GEmCherry constructs (GEmCherry in red; DAPI in blue; scale bar = 100 µm). (C) λ-scan showing that only the intensity but not the emission spectra changes upon mutating the GEmCherry sequence. (D) By insertion of 23 bp target sequence, the reading frame will be pushed to induce a premature stop-codon abolishing fluorescence. Cas9 targeting and induction of indels will move the sequence back in-frame and again allow for the expression of red fluorescent protein. (E) HEK293T cells transfected with out-of-frame GEmCherry constructs (GEmCherry in red; DAPI in blue; scale bar = 100 µm). (F) Quantification of fluorescence intensity of in-frame (solid color) and out-of-frame (striped) constructs. In-frame intensity of GEmCherry1 was significantly higher than GEmCherry2, 3, and 4 (p < 0.0001, n = 3) but so was the out-of-frame background fluorescence (p < 0.0001, n = 3). The in-frame intensity between GEmCherry3 and GEmCherry2, and 4 was also significantly different (p = 0.0068 and p = 0.0006, n = 3) but the out-of-frame background fluorescence was not. For GEmCherry2, 3, and 4 the out-of-frame background fluorescence was not significantly different than the no-plasmid control, based on the pixel density, although low background fluorescence was visibly observable in both GEmCherry 2, 3, and 4. One-way ANOVA with Tukey’s test were performed. Results are presented as mean with SD.
marginal improvements in GFP expression (Figure 4C). After in silico analysis of the Cas9 plasmid sequence we identified an ITR sequence from an AAV within the construct as the only distinct difference between the plasmids. Subsequent removal of this AAV sequence was shown to significantly restore transfection efficiency and expression in hESCs ($p = 0.0446$; Figures 4C,D), resulting in the final plasmid pCAG-SpCas9-2A-GFP-noITR. In parallel to transient transfection, we produced lentiviral particles containing Cas9 and sgRNA SORCS2-G2. This method proved the most rapid and efficient targeting method in hESCs. From the lentiviral-infected hESCs, 10 clones were expanded and three analyzed for indels in SORCS2 exon-1. Results showed that all three clones were successfully targeted and contained indels in SORCS2 and, interestingly, clone 9 displayed a heterozygous sequence (Figure 4E).

**DISCUSSION**

Gene editing in human pluripotent stem cells is challenging in comparison to mouse pluripotent stem cells which can be clonally expanded in the presence of LIF, facilitating the survival and selection of targeted clones. Homology-directed
repair methods have been improved significantly by the discovery of Cas9 and its development as a programmable nuclease system for targeted DSBs (Mali et al., 2013; Ran et al., 2013a). Despite these advancements, practical challenges of efficiently producing targeted clones remain. This study describes an optimized method for designing sgRNA sequences and an improved sgRNA delivery vector. Herein, we describe the development of a rapid assay system, GEmCherry2, which uses a single fluorescent reporter for assessing CRISPR Cas9 activity at sgRNA target sites. When compared with the TIDE assay, the GEmCherry2 assay showed a similar ranking of sequences (Brinkman et al., 2014). Additionally, and similar to the dual surrogate reporter system, the GEmCherry2 system can be used to assess ZFN, TALENS, DNA system efficiencies, or dual-nickase variants (Kim et al., 2011). Furthermore, an advantage of our reporter system over the dual fluorescent system is that it can be used in conjunction with a Cas9-GFP plasmid to evaluate Cas9 activity and overcome transfection efficiency discrepancies.
Gene editing in human embryonic stem cells. (A) Based on GEemCherry2 fluorescence we estimated the efficiency of three different sgRNAs (G1, G2, and G3) targeting exon1 of the SORCS2 gene. HEK293T cells were co-transfected with CRISPR Cas9-GFP and GEemCherry2 constructs and the Cas9 cutting efficiency was estimated based on high-intensity in-frame fluorescence (GEemCherry in red; GFP in green; scale bar = 100 µm). (B) The fluorescence intensity (measured as pixel density) was quantified as GEemCherry against GFP fluorescence and normalized against the out-of-frame background fluorescence. The fluorescence intensity was not significantly different between the G1, G2, and G3 sgRNA, although a trend was seen toward G2 generating more high-intensity RFP++ cells (One-Way ANOVA with Tukey’s test, n = 3, mean with SD).

(C) Images of hESCs stained against POU5F1, GFP, and DAPI (scale bar = 100 µm) after transfection with either pCAG-SpCas9-2A-GFP (top) or pCAG-SpCas9-2A-GFP-noITR (bottom). (D) Quantification of transfection efficiency and GFP expression before and after deletion of the AAV ITR from the pCAG-SpCas9-2A-GFP vector (*p = 0.0446; n_{ITR} = 6, n_{noITR} = 5). Unpaired two-tailed t-test was performed. Results are presented as mean with SD. (E) Sanger sequencing of the genomic region of H9 control and three H9-SORCS2 knock-out cell lines (clone 8, 9, and 10). The expected cut site of sgRNA SORCS2-G2 is marked in yellow.

more precisely. Using this system as a primary screen, we were able to identify a novel AAVS1 Cas9 active site with a low number of off-target sites and high Cas9 activity and we designed an efficient sgRNA for SORCS2. We attempted gene-targeting in hESCs and found that the majority of available CRISPR Cas9 plasmids contain an AAV ITR sequence in their
backbone that reduces transfection efficiency and transient expression. This silencing may be occurring in hESCs through a similar mechanism to what has been reported in other cell types, by which the ITR regions are rapidly identified and methylated by the host cell (Chanda et al., 2017). After removal of these sequences, a significant improvement in transfection efficiency and transient expression was obtained. Furthermore, as an alternative and more efficient method for Cas9-mediated knock-out of SORCS2, we used lentiviruses, which are highly efficient at infecting hESCs (Gropp et al., 2003). With this rapid delivery system, we produced 10 clones and selected three for subsequent screening of indels in the SORCS2 coding sequence. This approach proved to be highly efficient as all three clones showed indels in the region around the target site.

In addition to efficient sgRNA design, several methods exist for delivering Cas9 and sgRNA. The choice of delivery method will depend on the specific cell type and the application of either gene disruption or homologous recombination, which requires a repair template. Some of these methods include delivery of cell-penetrating Cas9 and guide RNA, capped RNA or integrase-deficient lentiviral vectors (Kim et al., 2014; Ramakrishna et al., 2014; Ortinski et al., 2017). In our approach, we used lentiviruses to infect hESCs. However, combining this with integrase-deficient or self-inactivating systems, such as Self-Limiting Cas9 circuit for Enhanced Safety and specificity (SLiCES) would be more desirable as they prevent the on-going expression of Cas9 (Petris et al., 2017).

Additional applications for GEmCherry2 also include assessing sgRNA activity against close matching off-target sequences in the genome or in patient lines with heterozygous single-nucleotide mutations. Sequences with multiple mismatches may be less problematic as modified version of Cas9 and a high-fidelity Cas9 that both have significantly reduced mismatch –NGG, –NAG, or –NGA (total/in-exon), and the number of mismatches and affected gene.

transfection efficiency, a reduced number of cells would be required when performing homologous directed repair in hPSCs, which currently requires large-scale production to identify correctly targeted clones.

**AUTHOR CONTRIBUTIONS**

MD: conceived and designed the experiments; CH, EÁ, KR, KG, SF, SH, MC, and FF: performed the experiments; JK: contributed instrumentation and expertise in the analysis; CH, and MD: analyzed data; MD and CH, wrote the manuscript. All authors reviewed the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00054/full#supplementary-material

**Figure S1** | Primers and oligos used for targeting the AA/1S1 locus (PPP1R12C) and validating CRISPR Cas9 activity at the target sites, AA/1S1 target sites C2, C3, C4, and T2, including PAM-sequence, used for designing sgRNA and GEmCherry target sequences. Regional primers to PCR the AA/1S1 locus from genomic DNA from cells transfected with Cas9 constructs. Sequencing primers used for TIDE analysis, designed for sequencing to start close to the Cas9 cut site (65–120 bp upstream of cut site), SORCS2 target sites G1, G2, and G3, including PAM-sequence (all in exon1). Regional primers to PCR the SORCS2 G2 target site from genomic DNA from knock-out H9 cell lines. The G2 fwd primer was also used for Sanger sequencing.

**Figure S2** | Evaluation of PPP1R12C sgRNA design and off-target effects using http://crispr.tefor.net/ with input PPP1R12C sequence: Homo sapiens (hg19), chr19:55628073-55628048, reverse genomic strand. The table shows the target sequence: (guide sequence + PAM), specificity score, predicted efficiency, out-of-frame score, number of off-targets for up to four mismatches flanked by motif –NGG, –NAG, or –NGA (total/in-exon), and the number of mismatches and affected gene.

**REFERENCES**


**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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