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NFIA is a gliogenic switch enabling rapid derivation of functional human astrocytes from pluripotent stem cells

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ABSTRACT

The mechanistic basis of gliogenesis, which occurs late in human development, is poorly understood. Here we identify nuclear factor IA (NFIA) as a molecular switch inducing human glial competency. Transient expression of NFIA is sufficient to trigger glial competency of human pluripotent stem cell-derived neural stem cells within 5 days and to convert these cells into astrocytes in the presence of glial-promoting factors, as compared to 3–6 months using current protocols. NFIA-induced astrocytes promote synaptogenesis, exhibit neuroprotective properties, display calcium transients in response to appropriate stimuli and engraft in the adult mouse brain. Differentiation involves rapid but reversible chromatin remodeling, glial fibrillary acidic protein (GFAP) promoter demethylation and a striking lengthening
of the G1 cell cycle phase. Genetic or pharmacological manipulation of G1 length partially mimics NFIA function. We
used the approach to generate astrocytes with region-specific or reactive features. Our study defines key mechanisms of the gliogenic switch and enables the rapid production of human astrocytes for disease modeling and regenerative medicine.

ATTACHMENTS

NFIA is a gliogenic switch enabling rapid derivation of functional human astrocytes from pluripotent stem cells.pdf

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PROTOCOL CITATION

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KEYWORDS

human astrocytes, pluripotent stem cells

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MATERIALS TEXT

Materials:

- N2 media
- NSC media
- Fibroblast growth factor 2
- HB-EGF [R&D Systems, No. 259-HE]
- Matrigel [BD Biosciences]
- ROCK inhibitor [No. Y-27632]
- LDN193189 [LDN, Stemgent]
- SB431542 [SB, Tocris; LSB]
- XAV939 [Stemgent]
- FGF2
- FUW-tetO-GFP [Addgene, No. 30130]
- NFIA
- SOX9
- FUCCI-O
- M2-rtTA [Addgene, No. 20342]
- psPAX2 [Addgene, No. 12260]
- pMD2.G [Addgene, No. 12259]
- X-tremeGene HP [Sigma]
- AMICON Ultra-15 Centrifugal Filter Units [Millipore]
- phosphate buffered saline [PBS]
- Cytofix/Cyto Perm kit [BD]
- Alexa 647-conjugated CD44 [Biolegend]
- unconjugated GFAP [Dako]
- Cyto Perm Buffer
- heparin-bound EGF [R&D Systems]
- leukemia inhibitory factor [Peprotech]
- paraformalaldehyde
- Alexa 488, Alexa 555 or Alexa 647 [Thermo]
- 4',6-diamidino-2-phenylindole [DAPI, Thermo]
- Precision Red [Cytoskeleton, Inc]
- IL-1α [Sigma]
- tumor necrosis factor [Cell Signaling Tech]
- C1q [MyBioSource]
- Human Complement C3 ELISA Kit [Abcam]
- Tris-HCl
- NaCl
- MgCl2
- Propidium iodine
- primary astrocytes [Sciencell]
- black ΔT dishes [Bioptechs]
- Fura-2 [Thermo]

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- HEPES
- CaCl2
- bovine serum albumin
- glutamate
- ATP
- KCl
- poly-l-ornithine/laminin/fibronectin dishes
- ascorbic acid
- EZ DNA Methylation-Direct Kit [Zymo]
- TOPO Zero Blunt vector [Invitrogen]
- EGTA
- Na-GTP
- Na2-phosphocreatine
- CsOH
- Tocris
- strychnine HCl [Sigma]
- tetrodotoxin [TTX, Alomone Labs]

Software:

- Axoscope software [Molecular Devices]
- Mini Analysis [Synaptosoft]
- FIJI [ImageJ]

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Cell culture

1 Maintain human pluripotent stem cells (both embryonic and induced) on vitronectin-coated dishes in Essential 8 (E8) medium (Thermo) as previously described.

2 Purchase induced PSCs.

3 Cells were used for differentiation between passages 30–50 and passaged twice every week. Cells were subjected to mycoplasma testing every **1344:00:00** - **2016:00:00** .

4 Maintain Neural stem cells, LTNSCs and glial progenitors on poly-l-ornithine/laminin/fibronectin-coated dishes in NSC medium consisting of N2 media with **10 µg/mL** Fibroblast growth factor 2 (FGF2), **10 µg/mL** epidermal growth factor (EGF) and 1:1,000 B27 supplement. LTNSCs were used between passages 15–20 and passaged every week.

5 Maintain Human pluripotent stem cell-derived astrocytes on poly-l-ornithine/laminin/fibronectin-coated dishes in astrocyte media consisting of N2 medium with **10 µg/mL of HB-EGF** (R&D Systems, No. 259-HE). After sorting, CD44-positive cells were passaged every week for 4 weeks and then every other week or until astrocyte processes started to detach.

6 Initially maintain commercial fetal astrocytes (Sciencell) in a commercial medium containing serum (Sciencell). Switch the serum-containing medium to N2 with **10 µg/mL HB-EGF** for at least two passages before performing the

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experiments.

7 Maintain human pluripotent stem cell-derived cortical excitatory neurons on poly-l ornithine / laminin / fibronectin-coated dishes in neurobasal medium with brain-derived neurotrophic factor (BDNF), ascorbic acid, glial cell line-derived neurotrophic factor (GDNF) and cyclic adenosine monophosphate. Change more than [M]**50 %** of the medium every week.

Differentation of hPSCs towards dorsal forebrain NSCs

8 Dorsal forebrain patterning. Dissociate human PSCs into single cells, and 2.5–3.0 × 10^5 cells per cm2 were plated onto Matrigel (BD Biosciences)-coated dishes in E8 containing [M]**10 Micromolar (µM) ROCK inhibitor** (No. Y-27632).

9 The following day (day 0), switch the cells to Essential 6 (E6) medium containing [M]**100 Micromolar (µM)** LDN193189 (LDN, Stemgent) and [M]**10 Micromolar (µM)** SB431542 (SB, Tocris; LSB).

10 Change medium every day for nine additional days (d8) as previously described.

11 To better promote an anterior forebrain fate (that is, for iPSCs), we add [M]**2 Micromolar (µM)** of XAV939 (Stemgent) in addition to LSB for **72:00:00** (d0–2) then from d3–8 maintained the cells in LSB without XAV939. 3d

Generation of cortical rosettes/NSCs 30m

12 From d8, dissociate the cells with Accutase for **00:30:00** at **37 °C** and passed through a 40 µm cell strainer. 30m

13 Resuspend the cells in N2 medium with brain-derived neurotrophic factor (BDNF), ascorbic acid, Sonic Hedgehog (SHH) and fibroblast growth factor 8 (FGF8) (N2-BASF), and plated at 5 × 10^5 cells on air-dried poly-l-ornithine/laminin/fibronect in-coated plates in **20 µl** droplets.

14 3d 0h 20m

Incubate the droplets at **37 °C** for **00:15:00** - **00:20:00** . N2-BASF8 medium + [M]**10 Micromolar (µM)** ROCKi medium was overlaid on the droplets and the medium changed every day. Rosette formation was expected within **48:00:00** - **72:00:00** (d12).

15 Following rosette formation, dissociate cells with Accutase and replated at relatively high density (3.5 × 10^5 cells per cm^2) to prevent spontaneous differentiation. Assay the cultured cells for correct regional patterning, differentiated or frozen.

Neuro-ectodermal differentiation of hPSCs towards ventral spinal cord

16 Similar to the dorsal forebrain, plate 2.5–3.0 × 10^5 cells per cm^2 onto Matrigel (BD Biosciences)-coated dishes in hPSC

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knockout serum replacement (KSR)-based medium containing [M]**10 Micromolar (µM)** ROCK inhibitor and [M]**10 µg/mL** FGF2.

17 The following day (day 0), switch the cells from a progressive gradient of KSR to N2 medium containing [M]**100 Micromolar (µM)** LDN193189 and [M]**10 Micromolar (µM)** SB431542 (LSB).

18 The following day (day 1), switch the medium to LSB with [M]**1 Micromolar (µM)** of retinoic acid and [M]**1 Micromolar (µM)** of purmorphamine and maintained for an additional **264:00:00** (d12). 1w 4d

19 Dissociate spinal cord progenitors with Accutase for **00:30:00** at **37 °C** and pass it through a 40 µm cell strainer. Plate the cells at 2.5×10^5 cells per cm^2 on poly-l-ornithine/laminin/fibronectin-coated plates and assayed for correct regional patterning, differentiated or frozen. 30m

Derivation of LTNSCs from hPSCs 10m

20 Initially, differentiated hPSCs using the method employed for dorsal forebrain NSCs. After initial regional patterning, differentiated cells were dissociated using [M]**10 %** dispase for **00:10:00** . 10m

21 Separated the cells into clumps and resuspend in N2 medium containing [M]**20 µg/mL** FGF2 and cultured in sterile, non-tissue culture-treated dishes.

22 Cells were expected to form a high number of neurospheres, and by 3-5 days neural rosette formation within the spheres should be apparent. Having purified the neurosphere cultures, they were landed on poly-l-ornithine / laminin / fibronectin-coated plates and cultured in N2 with [M]**10 µg/mL** FGF2, [M]**10 µg/mL** EGF and 1:1,000 B27 supplement (NSC media).

23 Observe Rosette-stage NSC outgrowth to confluency and then pass at high density (approximately 1:3) over 2-3 months.

24 Cells maintaining neuroepithelial morphology by passage 10 in NSC media were kept and analyzed for early NSC markers and differentiation potential.

Generation of NFIA- and SOX9-inducible constructs and lentiviral production and infection

25

Clone NFIA and SOX9 from cDNA using hPSC-derived astroglial progenitors (d90). Digest FUW-tetO-GFP (Addgene, No. 30130) with the restriction enzyme EcoRI to remove the GFP fragment, and either insert NFIA or SOX9 using traditional ligation cloning.

26 Plasmids containing NFIA, SOX9, FUCCI-O or M2-rtTA (Addgene, No. 20342), the packaging vector psPAX2 (Addgene, No. 12260) and transfect the envelope pMD2.G (Addgene, No. 12259) into 293 T cells X-tremeGene HP (Sigma) at a molar ratio of 1:2:1.

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27 Harvest virus at 48 and **72:00:00** post-transfection and concentrate it using AMICON Ultra-15 Centrifugal Filter Units (Millipore). 3d

28 Plate NSCs at 3.5 × 10^5 cells per cm^2 on poly-l-ornithine/laminin/fibronectin-coated dishes.

29  20h

Incubate the cells with viral particles generated (as described above) for **16:00:00** - **20:00:00** .

30 Switch the medium to NSC medium with **1 µg/mL** - **2 µg/mL** doxycycline and daily medium change for a minimum of **120:00:00** . 5d

31 

Detach cells using **0.05 %** trypsin and wash several times in preparation for CD44 labeling.

Intracellular FACS analysis and sorting

32 For both live and fixed sorting, dissociate cells using **0.05 %** trypsin and washed twice with phosphate buffered saline (PBS).

33 Fixed GFAP and CD44 analysis. 1h
Using the BD Cytofix/Cyto Perm kit (BD), resuspend 1 × 10^6 cells in **1 mL** Cytofix and placed **On ice** for **01:00:00** .

34 

Wash the cells twice with 1× Cyto Perm Buffer and resuspended in **100 µl** of 1× Cyto Perm Buffer.

35  40m

Add Alexa 647-conjugated CD44 (Biolegend) and unconjugated GFAP (Dako) to the cells, as described by the manufacturer, and incubate for **00:40:00** **On ice** .

36 

Wash the cells twice with 1× Cyto Perm Buffer and resuspended in **100 µl** of 1× Cyto Perm Buffer for secondary labeling.

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37 For labeling of GFAP, add Alexa 488 or 555 to the cells for **00:30:00** **On ice** . Wash cells twice with 1× Cyto Perm Buffer and submit for analysis. 30m

38 30m

Live CD44 sorting:
Resuspend approximately 4–5 × 10^6 cells in **100 µl** of sort buffer (**2 %** fetal bovine serum, **1 Milimolar (mM)** EDTA in PBS), and incubate CD44 conjugated with Alexa 647, as per the manufacturer's instructions, **On ice** for **00:20:00** - **00:30:00** .

39 Wash cells twice with sort buffer and submitted to the sorter.

40 Maintain CD44-positive cells in astrocyte induction medium (N2 with **10 µg/mL** heparin-bound EGF (R&D Systems) and **10 µg/mL** leukemia inhibitory factor (Peprotech) without doxycycline.

Immunohistochemistry 10m

41 Fix cells in PBS containing **4 %** paraformaldehyde for **00:10:00** , permeabilized using PBS with **0.5 %** Triton-X for **00:05:00** and stored in PBS with **0.2 %** Tween-20. 15m

42

Contain the blocking solution **5 %** donkey serum in PBS with **0.2 %** Tween-20. Dilute primary antibodies in the blocking solution and typically incubate **Overnight** at **4 °C** .

43 30m

Conjucate Secondary antibodies to either Alexa 488, Alexa 555 or Alexa 647 (Thermo) were added to the cells with incubation for **00:30:00** .

44 Identify nuclei by staining the cells with 4',6-diamidino-2-phenylindole (DAPI, Thermo). A list of antibodies used in this study is given in Supplementary Table 3.

Immunoblot

45 Harvest and lyse cells with RIPA buffer, and quantify protein using Precision Red (Cytoskeleton, Inc).

46 Load **10 µg** of protein to analyze protein expression. Full scans of blots for Fig. 2b (Supplementary

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Fig. 22), Fig. 4f (Supplementary Fig. 23), Fig. 4k (Supplementary Fig. 24) and Supplementary Fig. 7c (Supplementary Fig. 25) are available.

Gene expression and ATAC-sequencing analysis

47 RNA sequencing. Isolate RNA as previously described. Submit total RNA to the Memorial Sloan Kettering Cancer Center (MSKCC) Genomics Core for paired-end sequencing, aiming for 30–40 million reads.

48 Trim raw FASTQ files for adapters and aligned to the ENSEMBL GRCh38 genome build using STAR 2.5.0.

49 Generate and import matrices from the aligned files using HTSeq into DESeq2 for further analysis using a standard pipeline.

50 A list of the normalized read counts represented in Fig. 1 and Supplementary Fig. 8 can be found in Supplementary Table 1, and a list of genes expressed from all samples in Fig. 4 can be found in Supplementary Table 2.

51 ATAC sequencing:
Raw FASTQ files were aligned to the hg19 genome build using Bowtie2. Perform comparative analysis of alignment using the deepTools software package. Perform motif analysis and peak annotation using HOMER software and visualized using the IGV browser.

52 Upload all FASTQ files and Supplementary files to National Center for Biotechnology Information Gene Expression Omnibus under accession code GSE104232.

Cytokine treatment of human astrocytes 1d

53 Plate astrocytes at 2 × 10^4 cells per cm^2 and treated with [M]**3 µg/mL** IL-1α (Sigma), [M]**30 µg/mL** tumor necrosis factor (Cell Signaling Tech) and [M]**400 µg/mL** C1q (MyBioSource) for **24:00:00** . 1d

54 Isolate the medium and spun down to remove debris, and measure C3 levels using the Human Complement C3 ELISA Kit (Abcam) as per the manufacturer's instructions.

Cell cycle analysis

55 Isolate cells with dissociated nuclei using resuspension buffer ([M]**10 Milimolar (mM)** Tris-HCl, [M]**30 Milimolar (mM)** NaCl, [M]**20 Milimolar (mM)** MgCl2) and subsequently the resuspension buffer with [M]**1 %** NP-40 for cell cycle analysis.

56 Add propidium iodine ([M]**250 µg/mL**) to the cells followed by analysis with FACS.

57 Analyse a minimum of 10,000 events per condition. Data acquired were imported and analyzed by Flowjo software.

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Transplantation of NSCs, glial precursors and astrocytes into adult cortex

58 Perform all surgeries according to National Institutes of Health guidelines and approve it by the local Institutional Animal Care and Use Committee, the Institutional Biosafety Committee and the Embryonic Stem Cell Research Committee. A total of eight (or 20) [CF1] NOD-SCID IL2-Rgc null mice (20–35 g; Jackson Laboratory) received cell transplantation.

59 Anesthetize mice with isoflurane 5% at a maintenance flow rate of 2–3%. Transplant a total of 7×10^4 NFIA-induced astrocytes in **2 µl** through a **5 µl** Hamilton syringe at a rate of **1 µl/min** by an infusion pump attached to a stereotactic micromanipulator, into the genu of the corpus callosum (coordinates: AP +0.740, ML −1.00, DV −2.30 from bregma).

60 Transplant a total of 2×10^5 LTNSC **2 µl** into the subcortical gray matter, striatum (coordinates AP +0.500, ML −1.90, DV −3.20 from bregma).

61 Transplant a total of 7.5×10^4 H1-GFP-derived astrocytes in **2 µl** into the genu of the corpus callosum (coordinates: AP +0.740, ML −1.00, DV −2.30 from bregma).

62 Sacrify the mice at 1, 6 and 12 weeks after transplantation for immunohistochemical analysis.

Tissue processing

63 Euthanize mice were euthanized with an overdose of pentobarbital given intraperitoneally, then transcardially perfused with PBS followed by paraformaldehyde 4%.

64 Remove brains after gentle dissection, maintained overnight in 4% paraformaldehyde then soaked in 30% sucrose for 3d **48:00:00** - **72:00:00** .

65 Perform brain coronal sectioning (**030 µm** at **-20 °C**) by cryostat after embedding with Optimal Cutting Temperature (OCT) (Sakura Finetek).

Calcium imaging

66 Plate Human pluripotent stem cell-derived neural stem cells, astrocytes or primary astrocytes (Sciencell) onto poly-l-ornithine/laminin/fibronectin-coated **0.5 mm** black ΔT dishes (Bioptechs) and use for calcium imaging as previously described between days 60 and 120.

67 30m

Incubate Cultures with **5 Micromolar (µM)** Fura-2 (Thermo) for **00:30:00** at **37 °C** and dishes were mounted on a ΔT Heated Lid w/Perfusion system (Bioptechs).

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68 Perfuse cultures with normal Tyrode's solution (pH **7.4**) containing [M] **125 Milimolar (mM)** NaCl, [M] **5 Milimolar (mM)** KCl, [M] **25 Milimolar (mM)** glucose, [M] **25 Milimolar (mM)** HEPES, [M] **1 Milimolar (mM)** MgCl2, [M] **2 Milimolar (mM)** CaCl2 and [M] **0.1 %** (w/v) bovine serum albumin.

69 Supplement cultures with glutamate ([M] **100 Micromolar (µM)**), ATP ([M] **30 Micromolar (µM)**) or KCl ([M] **65 Milimolar (mM)**) for **00:01:00** and imaged every 30 s at 340 and 380 nm at a minimum of seven positions. 1m

70 Analyse time-lapse images using FIJI (ImageJ) by calculating the signal ratio between 380 and 340 nm.

Glutamate excitotoxicity assay

71 Derive cortical neurons by differentiating hPSCs towards a neuro-ectodermal fate (see above). Dissociate Neuro-ectodermal cells and replate to generate neural rosettes, and further differentiate into neurons by treatment with [M] **10 Micromolar (µM)** of a γ-secretase inhibitor (DAPT).

72 Replate neurons and assay for maturation markers or glutamate excitoxicity with or without astrocytes.

73 For glutamate excitotoxicity studies, plate 100,000 neurons per cm^2 on poly-l-ornithine/laminin/fibronectin dishes in N2 medium with BDNF, ascorbic acid and GDNF.

74 Add NFIA-induced astrocytes at 150,000 cells per cm^2 and co-culture for an additional **120:00:00** . 5d

75 Treat cells with **100 µm** or **500 µm** (final) l-glutamate for **01:00:00** in Hanks' buffered salt solution and recover in N2 medium with BDNF, ascorbic acid and GDNF. 1h

76 Add resazurin **48:00:00** after glutamate treatment to determine cell viability. 2d

Bisulfite conversion and sequencing 5d

77 Treat LTNSCs infected with NFIA with dox for **120:00:00** and sorte for CD44. 5d

78 Isolate cells were and perform bisulfite conversion using the EZ DNA Methylation-Direct Kit (Zymo) as described by the manufacturer.

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79 Primers for the regions of the GFAP STAT3 binding site were described previously.

80 Briefly, P1 and P2 correspond to −1,500 bp from the start site of GFAP (P1 forward: 5' AGGAGGGTTGTTTGTTTTTTAGAA, P1 reverse: 5' CCCTTCCTTATCTAACCTCCCTATA and P2 forward: 5' GTAGATT TGGTAGTATTGGGTTGGT, P2 reverse: 5' CCCTCACCCATTTATATCCTTAAA.

81 Amplify the GFAP promoter region using ZymoTaq Premix (Zymo) and cloned into the TOPO Zero Blunt vector (Invitrogen). Sent a minimum of ten colonies per condition for sequencing.

Electrophysiology

82 Perform whole-cell recordings as described previously, with slight modifications. Briefly, visualize neurons using a Zeiss microscope (Axioscope) equipped with a ×4 objective and a ×40 water immersion.

83 Record neurons at **23 °C** - **24 °C** .

84 Measure input resistance from voltage response elicited by intracellular injection of a current pulse (−100 pA, 200 ms).

85 Filter low-pass membrane voltage at 5 kHz and digitize at 10 kHz, using a Multiclamp 700B amplifier connected to a DigiData 1322 A interface (Axon Instruments) using Clampex 10.2 software (Molecular Devices). Calculate liquid junction potentials and correct offline.

86 During recording, perfuse neurons with freshly prepared artificial cerebrospinal fluid ([M]**126 Milimolar (mM)** NaCl, [M]**26 Milimolar (mM)** NaHCO3, [M]**3.6 Milimolar (mM)** KCl, [M]**1.2 Milimolar (mM)** NaH_2PO_4, [M]**1.5 Milimolar (mM)** MgCl2, [M]**2.5 Milimolar (mM)** CaCl2 and [M]**10 Milimolar (mM)** glucose), and saturate the solution with [M]**95 %** O_2– [M]**5 %** CO_2.

87 Pipette solution for all recordings contained [M]**140 Milimolar (mM)** CsCl, [M]**10 Milimolar (mM)** NaCl, [M]**10 Milimolar (mM)** HEPES, [M]**0.5 Milimolar (mM)** EGTA, [M]**3 Milimolar (mM)** Mg-ATP, [M]**0.2 Milimolar (mM)** Na-GTP and [M]**10 Milimolar (mM)** Na2-phosphocreatine, pH adjust to pH**7.3** with CsOH.

88 Add Bicuculline methochloride ([M]**20 Micromolar (µM)** , Tocris), [M]**1 Micromolar (µM)** strychnine HCl (Sigma) and [M]**0.5 Micromolar (µM)** tetrodotoxin (TTX, Alomone Labs) to the artificial cerebrospinal fluid for mEPSC recordings to block gamma-aminobutyric acid receptors, glycine receptors and Na+ channels, respectively.

89 Hold neurons at −80 mV and continuous recording of mEPSCs was made using Axoscope software (Molecular Devices).

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90 Perform data processing and analysis using MiniAnalysis (Synaptosoft) and Clampfit 10 (Molecular Devices). Detect Events by setting the threshold value, followed by visual confirmation of mEPSC detection.

91 Perform statistical analysis using Student's t-test or Mann–Whitney rank sum test as necessary, with a significant difference at $P < 0.05$. Data are expressed as mean ± standard error.

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