CELL STEM CELL

SUPPLEMENTARY INFORMATION

Zika Virus Infects Human Cortical Neural Precursors and Attenuates Their Growth

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SUPPLEMENTARY FIGURES AND LEGENDS

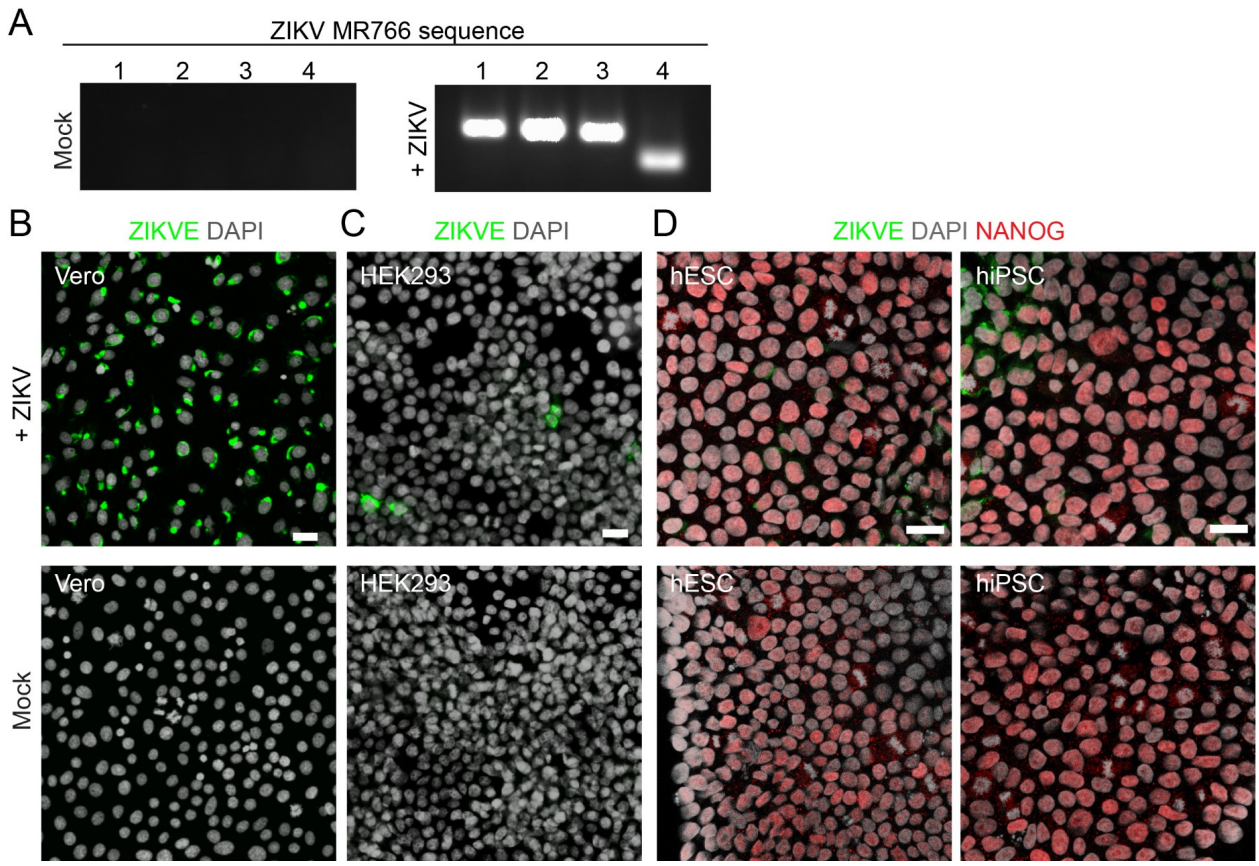

Figure S1, related to Figure 1.
(A) RT-PCR amplification of ZIKV genome from infected Vero cells. Primers were designed using the ZIKV MR766 sequence: #1: 89-279; #2: 4082-4281; #3: 1763-1952; #4: 1763-1850.
(B) Sample images of immunostaining of Vero cells with a pan-flavivirus anti-E antibody (ZIKVE) at 56 hours after infection or mock infection. Scale bar: 20 μm.
(C-D) Sample images of immunostaining of HEK293 cells (C) and hESCs (WA09) and hiPSCs (DF19-9-11T.H.; D) with ZIKVE 72 hours after ZIKV or mock infection. hESCs and hiPSCs were also stained with antibody against pluripotency marker NANOG. Note that ZIKVE$^+$ cells were located at the edge of the colonies and exhibited reduced levels of NANOG. Scale bars: 20 μm.

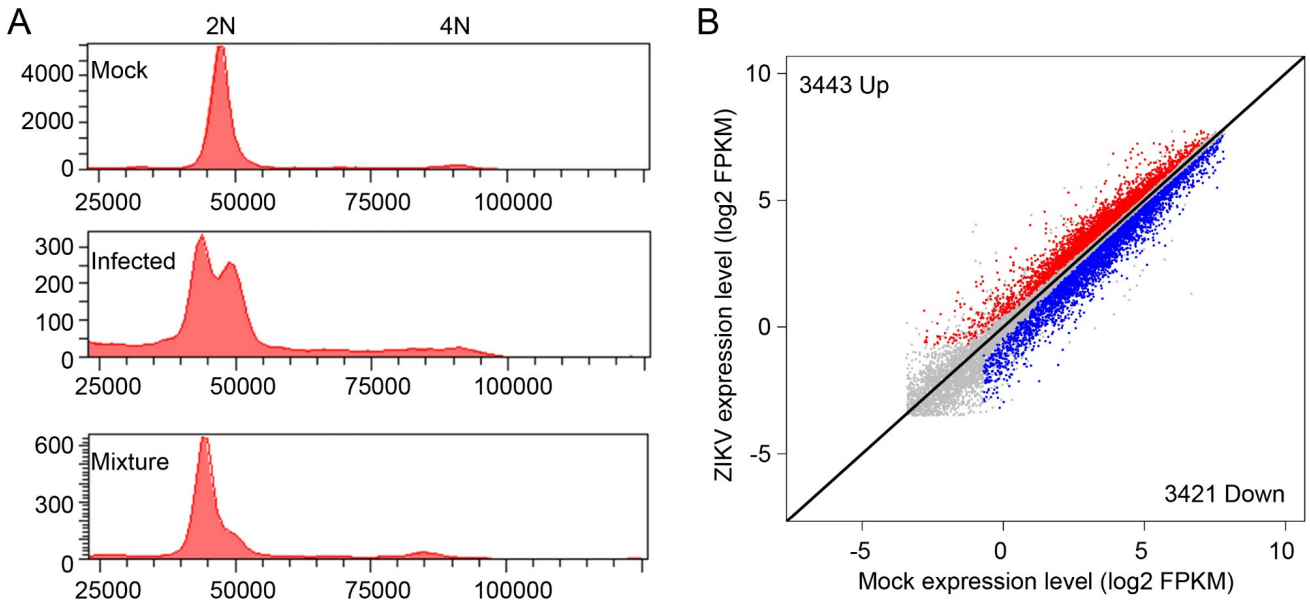

Figure S2, related to Figure 2.
(A) Sample flow cytometry plots of the distribution of hNPCs at different phases of cell cycle 72 hours after ZIKV or mock infection. An independent experiment using the same protocol as in Figure 2C.
(B) Scatter plot of global transcriptome changes between mock and ZIKV infected cells. Log2 FPKM (Fragments Per Kilobase of transcript per Million mapped reads) from RNA-seq data are plotted, and genes with significant differential expression values are highlighted. Upregulated genes are shown in red and downregulated genes are shown in blue.

SUPPLEMENTARY TABLES

Table S1. Summary of ZIKV infection of different cell types, related to Figure 1.

Name	Type	ZIKV permissiveness*
WA09	hESCs	+/-
DF19-9-11T.H.	hiPSCs	+/-
C1-2-NPC	hNPCs	+++
D3-2-NPC	hNPCs	+++
C1-2-N	differentiated immature neurons from hNPCs	+
293T	human embryonic kidney cell line	+/-
SNB-19	human CNS cell line (Glioblastoma)	+++
SF268	human CNS cell line (astrocytoma)	+++
Vero	monkey IFN^- kidney cell line	+++
C6/C36	mosquito (*Aedes albopictus)* cell line	+++

* +++: 65-100% cells infection after 3 days; +: 10-20% of the cells infected after 3 days; +/-: < 10% of cells infected after 3 days.

Table S2. Differential gene expression between ZIKV infected and mock infected hNPCs, related to Figure 2.
(A) Sequencing information
(B) List of downregulated genes
(C) List of upregulated genes

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Culture of Human iPSCs and Differentiation into Cortical Neural Progenitor Cells and Immature Neurons

Human iPSC lines were previously generated from skin biopsy samples of a male newborn (C1-2 line) and a male adult (D3-2 line) and had been fully characterized and passaged on MEF feeder layers (Wen et al., 2014). H9 hESCs (WA09 from WiCell) and hiPSCs (DF19-9-11T.H. from WiCell) were cultured under feeder-free conditions as described previously (Wu et al., 2012). All studies followed institutional IRB and ISCRO protocols approved by Johns Hopkins University School of Medicine and Florida State University. Human iPSCs (C1-2 and D3-2) were differentiated into forebrain-specific hNPCs and immature neurons following a previously established protocol (Wen et al., 2014). Briefly, hiPSCs colonies were detached from the feeder layer with 1 mg/ml collagenase treatment for 1 hour and suspended in embryonic body (EB) medium, consisting of FGF-2-free iPSC medium supplemented with 2 μM Dorsomorphin and 2 μM A-83, in non-treated polystyrene plates for 4 days with a daily medium change. After 4 days, EB medium was replaced by neural induction medium (NPC medium) consisting of DMEM/F12, N2 supplement, NEAA, 2 μg/ml heparin and 2 μM cyclopamine. The floating EBs were then transferred to matrigel-coated 6-well plates at day 7 to form neural tube-like rosettes. The attached rosettes were kept for 15 days with NPC medium change every other day. On day 22, the rosettes were picked mechanically and transferred to low attachment plates (Corning) to form neurospheres in NPC medium containing B27. The neurospheres were then dissociated with Accutase at 37ºC for 10 minutes and placed onto matrigel-coated 6-well plates at day 24 to form monolayer hNPCs in NPC medium containing B27. These hNPCs expressed forebrain-specific progenitor markers, including NESTIN, PAX6, EMX-1, FOXG1 and OTX2 (Wen et al., 2014). For neuronal differentiation, monolayer hNPCs were dissociated with Accutase at 37ºC for 5 minutes and placed onto Poly-D-Lysine/laminin-coated coverslips in the neuronal culture medium, consisting of Neurobasal medium supplemented with 2 mM L-glutamine, B27, cAMP (1 μM), L-Ascorbic Acid (200 ng/ml), BDNF (10 ng/ml) and GDNF (10 ng/ml) (Wen et al., 2014).

Preparation of ZIKV and Cell Infection

A ZIKV stock with the titer of $1x10^5$ Tissue Culture Infective Dose (TCID)/ml in the form of culture fluid from an infected rhesus *Macaca* cell line, LLC-MK2, was originally obtained from ZeptoMetrix (Buffalo, NY). This virus stock was then used to infect the *Aedes* C3/C36 cells at a MOI of 0.02. Supernatant from the infected mosquito cells was collected 4-6 days post-infection and frozen in aliquots for both titering and infection of human cells. An equal volume of culture medium from uninfected C3/C36 cells was used for mock infection. For all infections, 0.5-1 million cells were seeded into 12-well plates with or without coverlips one day before virus addition. hiPSCs (DF19-9-11T.H.) were cultured under feeder-free conditions in mTeSR medium before infection. All viral infection were performed under the same condition and the virus inoculum was removed after a 2-hour incubation and fresh medium for the appropriate cell type was added. To determine whether infected hNPCs produced more infectious ZIKV particles, supernatant from hNPC cultures 72 hours after infection was collected, filtered and then added to Vero cells for 48 hours.

RNA Extraction, RT-PCR and DNA sequencing

Total cellular RNA was purified from naïve or ZIKV-infected Vero cells using the Qiagen RNeasy Plus kit according to manufacturer's instructions. cDNA was produced from 1,000 ng total RNA, using random hexamers and an Invitrogen Superscript III first-strand kit according to the manufacturer's instructions. PCR was performed using GoTaq green PCR master mix (Promega) and Zika MR766 -genome specific primers (genome position 80-279: forward, TGGGAGGTTTGAAGAGGCTG; reverse, TCTCAACATGGCAGCAAGATCT; 1763-1850, forward, CATATTCCTTGTGCACCGCG, reverse, GCATACTGCACCTCCACTGT; 1763-1952, forward, CATATTCCTTGTGCACCGCG, reverse, TCAGTAATCACGGGGTTGGC; 4082-5456, forward,

CAAGGAGTGGGAAGCGGAG, reverse, CCATGTGATGTCACCTGCTCT; 5456-5620, forward, GCGATGCGTTTCCAGATTCC, reverse, TTGTCAGACAGGCTGCGATT). PCR products were purified and directly sequenced without cloning.

Immunocytochemistry
Cells were fixed with 4% paraformaldehyde (Sigma) for 15 min at room temperature. Samples were permeabilized and blocked with 0.25% Triton X-100 (Sigma) and 10% donkey serum in PBS for 20 min as previously described (Chiang et al., 2011; Wen et al., 2014; Yoon et al., 2014). Samples were then incubated with primary antibodies at 4□C overnight, followed by incubation with secondary antibodies for 1 hr at room temperature. The following antibodies were used: anti-Flavivirus Group Antigen Antibody (clone D1-4G2-4-15; mouse; 1:500; Millipore), anti-Cleaved caspase-3 (Asp15; Rabbit; 1:500; Cell Signaling Technology), anti-NANOG (goat; 1:500; R & D Systems). Antibodies were prepared in PBS containing 0.25% Triton X-100 and 10% donkey serum. Images were taken by Zeiss LSM 880 confocal microscope, or Zeiss Axiovert 200M microscope.

Flow Cytometry Analysis
ZIKV-infected hNPCs at 72 hours post-infection and mock-infected parallel cultures were fixed for DNA content analysis. Staining with a Propidium Iodide Flow Cytometry Kit for Cell Cycle Analysis (Abcam) was performed according to manufacturer's instructions. Cell cycle progression data were obtained using BD FACS Canto Ruo Special Order System flow cytometer (Becton Dickinson) and analyzed using FACS Diva software.

Transcriptome Analyses
ZIKV-infected hNPCs 56 hours after ZIKA and mock infection in parallel cultures were used for global transcriptome analysis. RNA-seq libraries were generated from duplicated samples per condition using the Illumina TruSeq RNA Sample Preparation Kit v2 following manufacturer’s protocol. An Agilent 2100 BioAnalyzer and DNA1000 kit (Agilent) were used to quantify amplified cDNA, and a qPCR-based KAPA library quantification kit (KAPA Biosystems) was used to accurately quantify library concentration. 12 pM diluted libraries were used for sequencing. 75-cycle paired-end sequencings were performed using Illumina MiSeq and single-end sequencings were performed as technical replicates using Illumina NextSeq (Table S2A). Image processing and sequence extraction were performed using the standard Illumina Pipeline (BaseSpace). RNA-seq reads were aligned using tophat v2.0.13 (Trapnell et al., 2012). Significantly differentially expressed genes were identified using cuffdiff by comparing FPKMs between all pairs of samples with P value < 0.05 (Trapnell et al., 2012) (Figure S2B and Table S2B-C). Gene Ontology analyses on biological process were performed by The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang da et al., 2009).

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