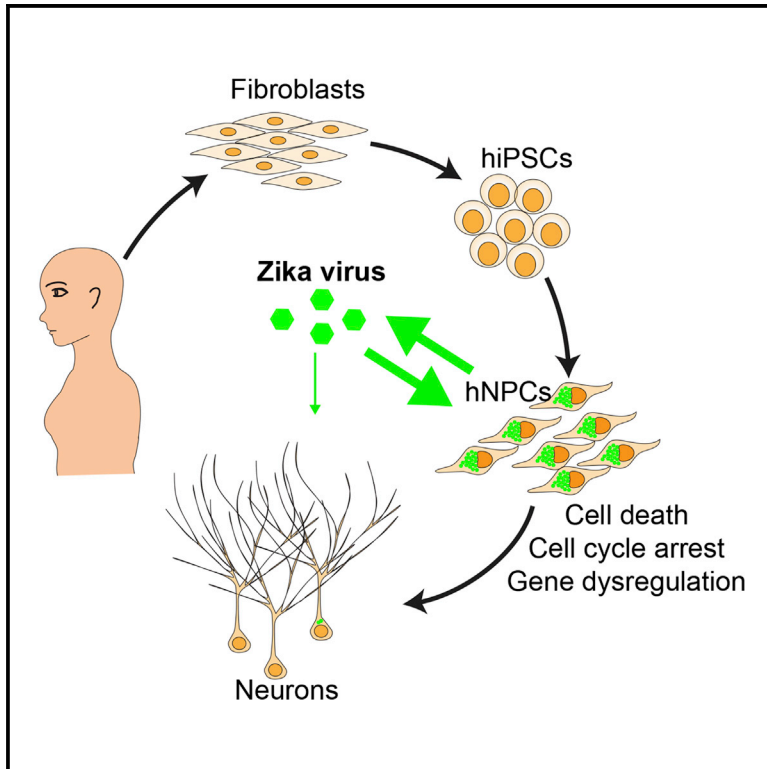# Cell Stem Cell

Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth

Graphical Abstract

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In Brief

The suspected link between ZIKV infection and microcephaly is an urgent global health concern. Tang et al. report that ZIKV virus directly infects human cortical neural progenitor cells with high efficiency, resulting in stunted growth of this cell population and transcriptional dysregulation.

Highlights

- Zika virus (ZIKV) infects human embryonic cortical neural progenitor cells (hNPCs)

- ZIKV-infected hNPCs produce infectious ZIKV particles

- ZIKV infection leads to increased cell death of hNPCs

- ZIKV infection dysregulates cell cycle and transcription in hNPCs

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Brief Report

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Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth

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SUMMARY

The suspected link between infection by Zika virus (ZIKV), a re-emerging flavivirus, and microcephaly is an urgent global health concern. The direct target cells of ZIKV in the developing human fetus are not clear. Here we show that a strain of the ZIKV, MR766, serially passaged in monkey and mosquito cells efficiently infects human neural progenitor cells (hNPCs) derived from induced pluripotent stem cells. Infected hNPCs further release infectious ZIKV particles. Importantly, ZIKV infection increases cell death and dysregulates cell-cycle progression, resulting in attenuated hNPC growth. Global gene expression analysis of infected hNPCs reveals transcriptional dysregulation, notably of cell-cycle-related pathways. Our results identify hNPCs as a direct ZIKV target. In addition, we establish a tractable experimental model system to investigate the impact and mechanism of ZIKV on human brain development and provide a platform to screen therapeutic compounds.

Zika virus (ZIKV), a mosquito-borne flavivirus, is now reported to be circulating in 26 countries and territories in Latin America and the Caribbean (Petersen et al., 2016). While infected individuals can often be asymptomatic or have only mild symptoms, of mounting concern are reports linking ZIKV infection to fetal and newborn microcephaly and serious neurological complications, such as Guillain-Barré syndrome (Petersen et al., 2016). The World Health Organization declared a Public Health Emergency of International Concern on February 1 of 2016 (Heymann et al., 2016). ZIKV infects human skin cells, consistent with its

major transmission route (Hamel et al., 2015). ZIKV was detected in the amniotic fluid of two pregnant women whose fetuses had been diagnosed with microcephaly (Calvet et al., 2016), suggesting that ZIKV can cross the placental barrier. ZIKV was also found in microcephalic fetal brain tissue (Mlakar et al., 2016). Because so little is known about direct cell targets and mechanisms of ZIKV, and because access to fetal human brain tissue is limited, there is an urgent need to develop a new strategy to determine whether there is a causal relationship between ZIKV infection and microcephaly. Here we used human induced pluripotent stem cells (hiPSCs) as an in vitro model to investigate whether ZIKV directly infects human neural cells and the nature of its impact.

We obtained a ZIKV stock from the infected rhesus *Macaca* cell line LLC-MK2. We passaged the virus in the mosquito C6/C36 cell line and titered collected ZIKV on Vero cells, an interferon-deficient monkey cell line commonly used to titer viruses. Sequences of multiple RT-PCR fragments generated from this stock (Figure S1A) matched the sequence of MR766, the original ZIKV strain that likely passed from an infected rhesus monkey to mosquitos (Dick et al., 1952). We first tested several human cell lines and found varying levels of susceptibility to ZIKV infection (Table S1). Notably, the human embryonic kidney cell line HEK293T showed low permissiveness for ZIKV infection (Figure S1C).

To identify direct target cells of ZIKV in the human neural lineage, we used a highly efficient protocol to differentiate hiPSCs into forebrain-specific human neural progenitor cells (hNPCs), which can be further differentiated into cortical neurons (Wen et al., 2014). The titer of ZIKV in the infected humans is currently unknown. We performed infections at a low multiplicity of infection (MOI < 0.1) and the medium containing virus inoculum was removed after a 2 hr incubation period. Infection rates were then quantified 56 hr later with RT-PCR using MR766-specific primers (Figure S1A) and with immunocytochemistry using an anti-ZIKV envelope antibody (Figures 1A and 1B). The hNPCs
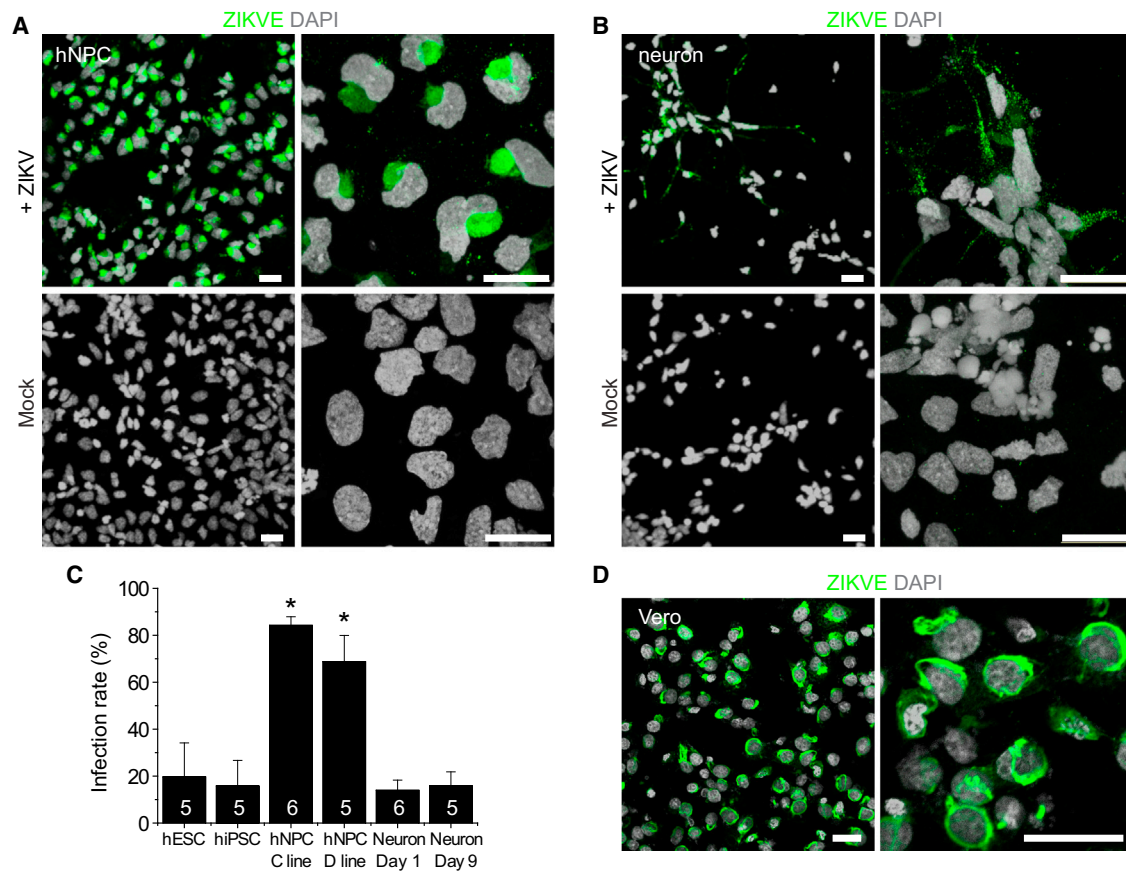
Figure 1. ZIKV Infects hiPSC-Derived Neural Progenitor Cells with High Efficiency

(A and B) Sample confocal images of forebrain-specific hNPCs (A) and immature neurons (B) 56 hr after infection with ZIKV supernatant, immunostained for ZIKV envelop protein (ZIKVE; green) and DAPI (gray). Cells were differentiated from the C1-2 hiPSC line. Scale bars, 20 μm.

(C) Quantification of infection efficiency for different cell types, including hESCs, hiPSCs, hNPCS derived from two different hiPSCs, and immature neurons 1 or 9 days after differentiation from hNPCs. Both hESCs and hiPSCs were analyzed 72 hr after infection, whereas all other cells were analyzed 56 hr after infection. Numbers associated with bar graphs indicate numbers of independent experiments. Values represent mean ± SD (*p < 0.01; Student's t test).

(D) Production of infectious ZIKV particles by infected hNPCs. Supernatant from hNPC cultures 72 hr after ZIKV infection was collected and added to Vero cells for 2 hr. The Vero cells were further cultured for 48 hr. Shown are sample images of ZIKVE immunostaining (green) and DAPI (gray). Scale bars, 20 μm.

See also Figure S1 and Table S1.

were readily infected by ZIKV in vitro, with the infection spreading to 65%–90% of the cells within 3 days of inoculation (Figures 1A and 1C). Quantitative analysis showed similar results for hNPCs derived from hiPSC lines of two different subjects (Figure 1C). As a control, we also exposed human embryonic stem cells (hESCs), hiPSCs, and immature cortical neurons to ZIKV under the same conditions. hESCs and hiPSCs could also be infected by ZIKV, but the infection was limited to a few cells at the colony edge with reduced expression of the pluripotent marker NANOG (Figures 1C and S1D; Table S1). Immature neurons differentiated from hNPCs also exhibited lower levels of infection under our conditions (Figures 1B and 1C). Together, these results establish that hNPCs, a constitutive population of the developing embryonic brain, are a direct cell target of ZIKV.

ZIKV envelope immunostaining exhibited the characteristic intracellular "virus factory" pattern of flaviviruses (Romero-Brey and Bartenschlager, 2014) (Figure 1A). We therefore tested infectivity using supernatant from infected hNPCs and observed robust infection of Vero cells (Figure 1D), indicating that produc-

tive infection of hNPCs leads to efficient secretion of infectious ZIKV particles.

We next determined the potential impact of ZIKV infection on hNPCs. We found a 29.9% ± 6.6% reduction in the total number of viable cells 66–72 hr after ZIKV infection, as compared to the mock infection (n = 3). Interestingly, ZIKV infection led to significantly higher caspase-3 activation in hNPCs 3 days after infection, as compared to the mock infection, suggesting increased cell death (Figures 2A and 2B). Furthermore, analysis of DNA content by flow cytometry suggested cell-cycle perturbation of infected hNPCs (Figures 2C and S2A). Therefore, ZIKV infection of hNPCs leads to attenuated growth of this cell population that is due, at least partly, to both increased cell death and cell-cycle dysregulation.

To investigate the impact of ZIKV infection on hNPCs at the molecular level, we employed global transcriptome analyses (RNA-seq). Our genome-wide analyses identified a large number of differentially expressed genes upon viral infection (Figure S2B and Table S2). Gene Ontology analyses revealed a particular
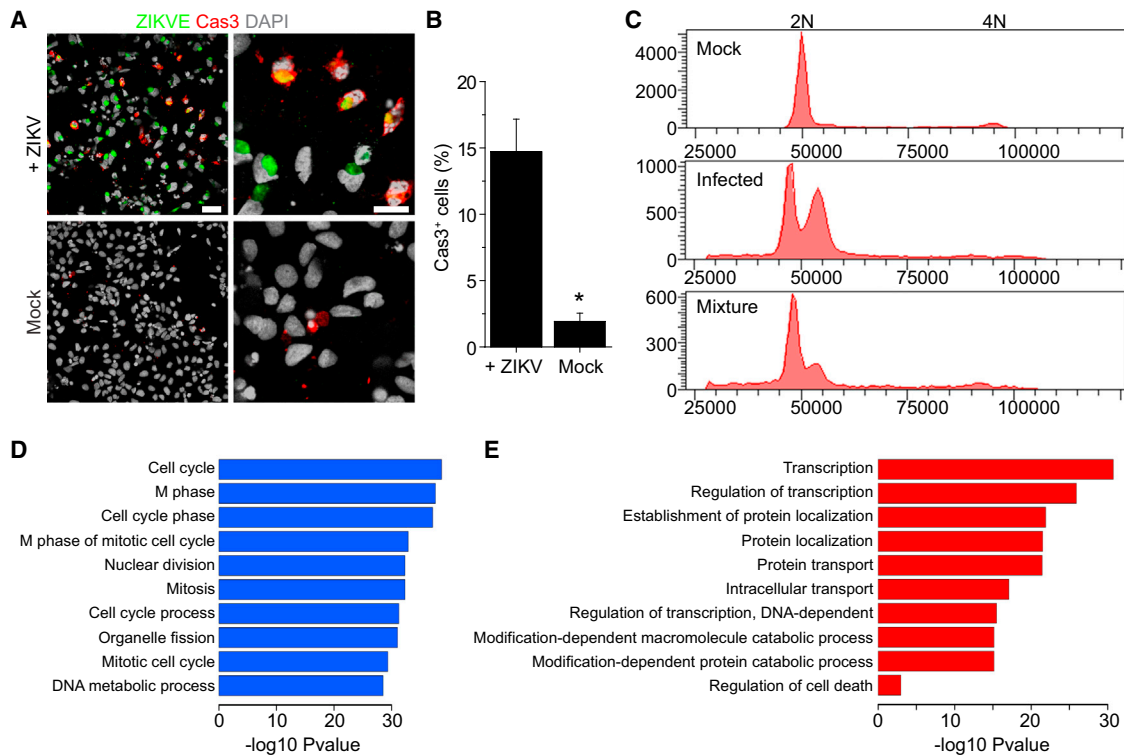
Figure 2. ZIKV-Infected hNPCs Exhibit Increased Cell Death and Dysregulated Cell-Cycle Progression and Gene Expression

(A and B) Increased cell death of ZIKV-infected hNPCs. Shown in (A) are sample images of immunostaining of hNPCs for ZIKVE (green) and cleaved-caspase-3 (Cas3; red) and DAPI (gray) 72 hr after ZIKV infection. Scale bars, 20 μm. Shown in (B) is the quantification. Values represent mean ± SEM (n = 6; *p < 0.01; Student's t test).

(C) Cell-cycle perturbation of hNPCs infected by ZIKV. Shown are sample flow cytometry analyses of distributions of hNPCs (from the C1-2 line) at different phases of the cell cycle 72 hr after ZIKV or mock infection. For the mixture sample, mock and infected hNPCs were mixed at a ratio of 1:1 following propidium iodide staining of each sample.

(D and E) RNA-seq analysis of hNPCs (C1-2 line) 56 hr after ZIKV or mock infection. Genes with significant differences in expression between infected and uninfected hNPCs were subjected to GO analyses. The top 10 most significant terms are shown for downregulated (D) and upregulated (E) genes, respectively. The −log10 p values are indicated by bar plots. An additional term of regulation of programmed cell death is also shown for upregulated genes (E). See also Figure S2 and Table S2.

enrichment of downregulated genes in cell-cycle-related pathways (Figure 2D), which is consistent with our flow cytometry findings (Figure 2C). Upregulated genes were primarily enriched in transcription, protein transport, and catabolic processes (Figure 2E). Consistent with increased caspase-3 activation observed by immunocytochemistry (Figures 2A and 2B), RNA-seq analysis revealed upregulation of genes, including caspase-3, involved in the regulation of the apoptotic pathway (Figure 2E). These global transcriptome datasets not only support our cell biology findings but also provide a valuable resource for the field.

It is not known whether specific strains of ZIKV circulating in geographically diverse parts of the world differ in their ability to impact neural development, and the stain we used had been discovered prior to the current reports of a potential epidemiologic link between ZIKV and microcephaly. Nevertheless, our results clearly demonstrate that ZIKV can directly infect hNPCs in vitro with high efficiency and that infection of hNPCs leads to attenuated population growth through virally induced caspase-3-mediated apoptosis and cell-cycle dysregulation. Infected hNPCs also release infectious viral particles, which pre-

sents a significant clinical challenge for developing effective therapeutics to arrest or block the impact of infection. Future studies using the hiPSC/hNPC model can determine whether various ZIKV strains impact hNPCs differently and, conversely, whether a single ZIKV strain differentially affects hNPCs from hiPSCs of various human populations.

Flaviviruses tend to have broad cellular tropisms and multiple factors contribute to pathogenic outcomes, including specific cellular response and tissue accessibility. Dengue virus infects cells of several lineages and hematopoietic cells play an essential role in the associated pathogenesis (Pham et al., 2012). West Nile virus infects epithelial cells of multiple tissues and can be neuroinvasive (Suthar et al., 2013). We note that ZIKV also infects other human cell types, including skin cells and fibroblasts (Hamel et al., 2015), and it remains unknown how ZIKV may gain access to the fetal brain (Mlakar et al., 2016). The capacity of ZIKV to infect hNPCs and attenuate their growth underscores the urgent need for more research into the role of these cells in putative ZIKV-related neuropathology. The finding that ZIKV also infects immature neurons raises critical questions about pathological effects on neurons and other neural cell types in

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the brain, as well as potential long-term consequences. Intriguingly, an early animal study showed ZIKV infection of neurons and astrocytes in mice and observed enlarged astrocytes (Bell et al., 1971). Our study also raises the question of whether ZIKV infects neural stem cells in adult humans (Bond et al., 2015).

In summary, our results fill a major gap in our knowledge about ZIKV biology and serve as an entry point to establish a mechanistic link between ZIKV and microcephaly. Our study also provides a tractable experimental system for modeling the impact of ZIKV on neural development and for investigating underlying cellular and molecular mechanisms. Of equal importance, our hNPC model and robust cellular phenotype comprise a readily scalable platform for high-throughput screens to prevent ZIKV infection of hNPCs and to ameliorate its pathological effects during neural development.

ACCESSION NUMBERS

The accession number for RNA-seq data reported in this paper is GEO: GSE78711.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes two figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2016.02.016.

AUTHOR CONTRIBUTIONS

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