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SUPPLEMENTARY MATERIALS

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ZIKA VIRUS

Zika virus impairs growth in human neurospheres and brain organoids

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Since the emergence of Zika virus (ZIKV), reports of microcephaly have increased considerably in Brazil; however, causality between the viral epidemic and malformations in fetal brains needs further confirmation. We examined the effects of ZIKV infection in human neural stem cells growing as neurospheres and brain organoids. Using immunocytochemistry and electron microscopy, we showed that ZIKV targets human brain cells, reducing their viability and growth as neurospheres and brain organoids. These results suggest that ZIKV abrogates neurogenesis during human brain development.

Primary microcephaly is a severe brain malformation characterized by the reduction of the head circumference. Patients display a heterogeneous range of brain impairments that compromise motor, visual, hearing, and cognitive functions (1).

Microcephaly is associated with decreased neuronal production as a consequence of proliferative defects and death of cortical progenitor cells (2). During pregnancy, the primary etiology of microcephaly varies from genetic mutations to external insults. The so-called TORCHS factors (toxoplasmosis, rubella, cytomegalovirus, herpes virus, and syphilis) are the main congenital infections that compromise brain development in utero (3).

An increase in the rate of microcephaly in Brazil has been associated with the recent outbreak of Zika virus (ZIKV) (4, 5), a flavivirus that is transmitted by mosquitoes (6) and sexually (7–9). So far, ZIKV has been described in the placenta and amniotic fluid of microcephalic fetuses (10–13) and in the blood of microcephalic newborns (11, 14). ZIKV had also been detected within the brain of a microcephalic fetus (13, 14), and recently, direct evidence has emerged that ZIKV is able to infect and cause the death of neural stem cells (15).

We used human induced pluripotent stem (iPS) cells cultured as neural stem cells (NSCs), neurospheres, and brain organoids to explore the consequences of ZIKV infection during neurogenesis and growth with three-dimensional culture models. Human iPS-derived NSCs were exposed to ZIKV [multiplicity of infection (MOI), 0.25 to 0.0025]. After 24 hours, ZIKV was detected in NSCs (Fig. 1, A to D); viral envelope protein was evident in 10.10% (MOI, 0.025) and 21.7% (MOI, 0.25) of cells exposed to ZIKV (Fig. 1E). Viral RNA was also detected in the supernatant of infected NSCs (MOI, 0.0025) by quan-

titative reverse transcriptase polymerase chain reaction (qRT-PCR) (Fig. 1F), providing evidence of productive infection.

To investigate the effects of ZIKV during neural differentiation, mock- and ZIKV-infected NSCs were cultured as neurospheres. After 3 days in vitro (DIV), mock-infected NSCs generated round neurospheres. However, ZIKV-infected NSCs generated neurospheres with morphological abnormalities and cell detachment (Fig. 2B). After 6 DIV, hundreds of neurospheres grew under mock conditions (Fig. 2, C and E). In ZIKV-infected NSCs (MOI, 2.5 to 0.025), only a few neurospheres survived (Fig. 2, D and E).

Mock-infected neurospheres presented the expected ultrastructural morphology of the nucleus and mitochondria (Fig. 3A). Viral particles were present in ZIKV-infected neurospheres, similar to those observed in murine glial and neuronal cells (16). ZIKV was bound to the membranes and observed in mitochondria and vesicles of cells within infected neurospheres (arrows in Fig. 3, B and F). Apoptotic nuclei, a hallmark of cell death, were observed in all ZIKV-infected neurospheres that we analyzed (Fig. 3B). ZIKV-infected cells in neurospheres presented smooth membrane structures (Fig. 3, B and F), similar to other cell types infected with dengue virus (17). These results suggest that ZIKV induces cell death in human neural stem cells and thus impairs the formation of neurospheres.

To further investigate the impact of ZIKV infection during neurogenesis, human iPS-derived brain organoids (18) were exposed to ZIKV and observed for 11 DIV (Fig. 4). The growth rates of 12 individual organoids (six mock- and six ZIKV-infected) were measured during this period (Fig. 4, A to D). As a result of ZIKV infection, the average growth area of ZIKV-exposed organoids was reduced by 40% compared with brain organoids under mock conditions [$0.624 \pm 0.064 \text{ mm}^2$ for ZIKV-exposed organoids versus $1.051 \pm 0.1084 \text{ mm}^2$ for mock-infected organoids (normalized); Fig. 4E].

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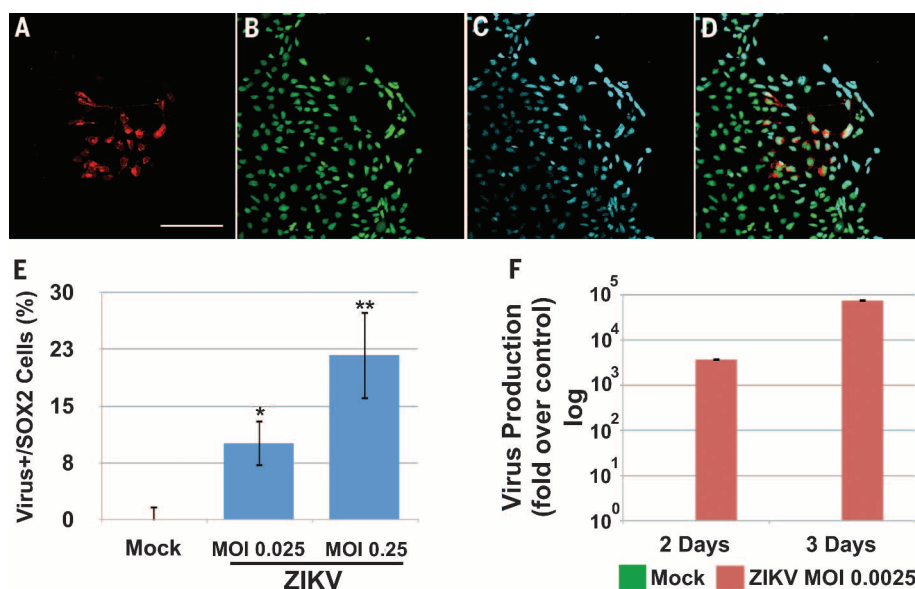


Fig. 1. ZIKV infects human NSCs. Shown are confocal microscopy images of iPS-derived NSCs double-stained for (A) ZIKV in the cytoplasm and (B) SOX2 in the nuclei, 1 day after virus infection. (C) DAPI (4',6-diamidino-2-phenylindole) nuclear staining. (D) Merged channels show perinuclear localization of ZIKV (red). Scale bar, 100 μ m. (E) Percentage of ZIKV-infected SOX2-positive cells (MOI, 0.25 and 0.025). (F) qRT-PCR analysis of ZIKV RNA extracted from supernatants of mock- and ZIKV-infected neurospheres (MOI, 0.0025) after 3 DIV, showing amplification only in infected cells. Virus production was normalized to 12-hour postinfection controls. Data are presented as means \pm SEM ($n = 5$). * $P < 0.05$; ** $P < 0.01$; Student's t test.

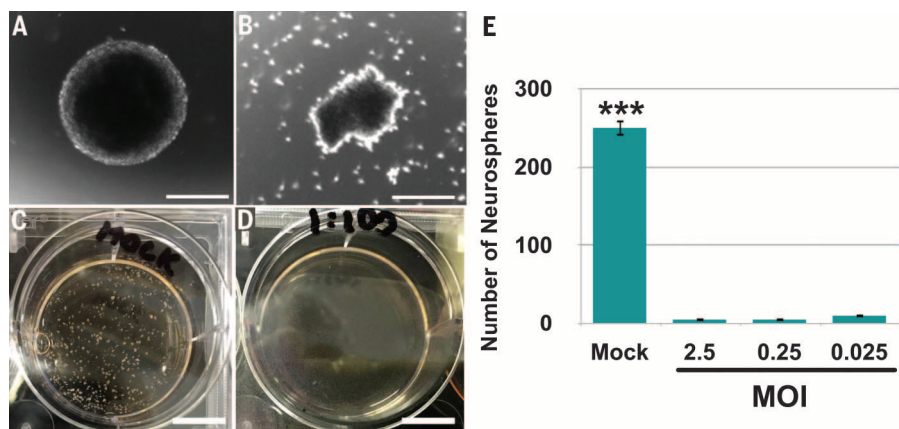


Fig. 2. ZIKV alters morphology and halts the growth of human neurospheres. (A) A control neurosphere displays spherical morphology after 3 DIV. (B) An infected neurosphere shows morphological abnormalities and cell detachment after 3 DIV. (C) A culture well plate containing hundreds of mock-infected neurospheres after 6 DIV. (D) A well plate containing few ZIKV-infected neurospheres (MOI, 2.5 to 0.025) after 6 DIV. Scale bars, 250 μ m in (A) and (B) and 1 cm in (C) and (D). (E) The number of neurospheres at different MOI. Data are presented as means \pm SEM ($n = 3$). *** $P < 0.01$; Student's t test.

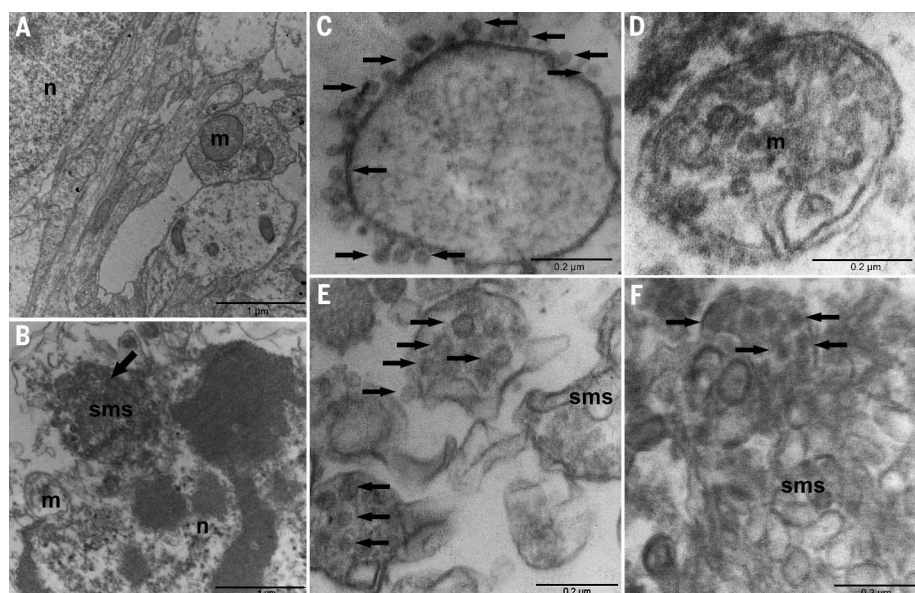
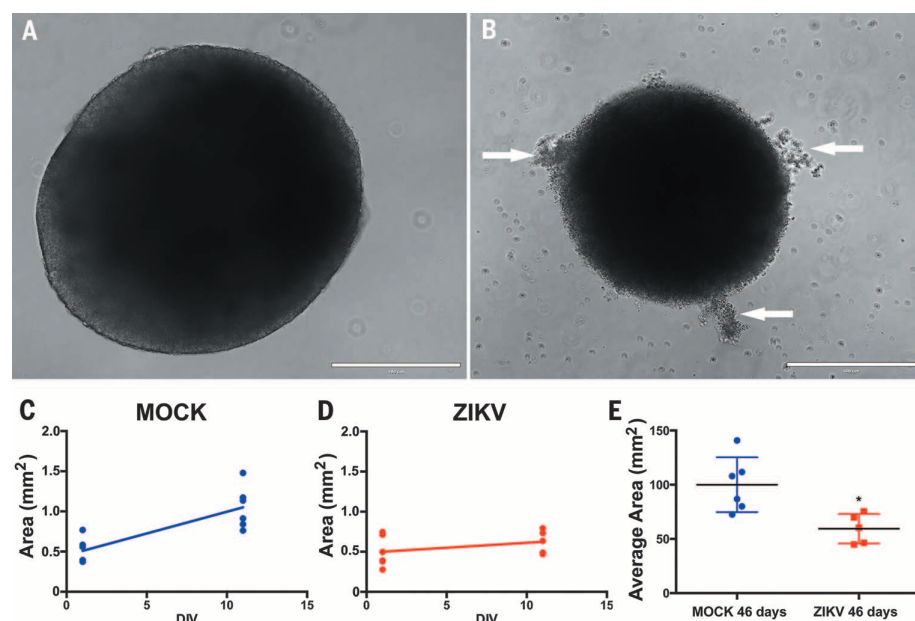


Fig. 3. ZIKV induces death in human neurospheres. These micrographs show the ultrastructure of mock- and ZIKV-infected neurospheres after 6 DIV. (A) Mock-infected neurosphere showing cell processes and organelles. (B) ZIKV-infected neurosphere showing a pyknotic nucleus, swollen mitochondria, smooth membrane structures, and viral envelopes (arrow). (C) Viral envelopes on the cell surface (arrows). (D) Swollen mitochondria. (E) Viral envelopes inside the endoplasmic reticulum (arrows). (F) Viral envelopes close to smooth membrane structures (arrows). Scale bars, 1 μ m in (A) and (B) and 0.2 μ m in (C) to (F). m, mitochondria; n, nucleus; sms, smooth membrane structures.

Fig. 4. ZIKV reduces the growth rate of human brain organoids. Brain organoids 35 days old were exposed to (A) mock conditions or (B) ZIKV for 11 DIV. ZIKV-infected brain organoids show reduced growth compared with the mock-infected controls. Arrows point to detached cells. Organoid area was measured before and after 11 DIV of exposure to (C) mock conditions or (D) ZIKV. Plotted lines represent the growth rate. (E) The average area of 46-DIV brain organoids, 11 DIV after mock or ZIKV infection. Data are presented as means (black bars) \pm SEM ($n = 6$). * $P < 0.05$; Student's t test.



We used cells infected with dengue virus 2 (DENV2), a flavivirus with genetic similarities to ZIKV (11, 19), as a second control group in addition to the mock infection group. One day after viral exposure, DENV2 infected human NSCs at a similar rate as that of ZIKV (fig. S1, A and B). However, after 3 DIV, there was no increase in caspase 3/7-mediated cell death induced by DENV2 at the same MOI of 0.025 that was used for ZIKV infection (fig. S1, C and D). In contrast, ZIKV induced caspase 3/7-mediated cell death in NSCs, consistent with the results described by Tang and colleagues (15). After 6 DIV, cell viability significantly differed between ZIKV-exposed NSCs and DENV2-exposed NSCs (fig. S1, E and F). In addition, neurospheres exposed to DENV2 displayed a round morphology similar to that of uninfected neurospheres after 6 DIV (fig. S1G). Last, there was no reduction of growth in brain organoids that were exposed to DENV2 for 11 days, relative to those grown under mock conditions [$1.023 \pm 0.1308 \text{ mm}^2$ for DENV2-infected organoids versus $1.011 \pm 0.2471 \text{ mm}^2$ for mock-infected organoids (normalized); fig. S1, H and I]. These results suggest that the deleterious consequences of ZIKV infection in human NSCs, neurospheres, and brain organoids are not a general feature of the flavivirus family. Neurospheres and brain organoids are complementary models for studying embryonic brain development in vitro (20, 21). Whereas neurospheres present the very early characteristics of neurogenesis, brain organoids recapitulate the orchestrated cellular and molecular early events comparably to the first-trimester fetal neocortex, including gene expression and cortical layering (18, 22). Our results demonstrate that ZIKV induces cell death in human iPS-derived NSCs, disrupts the formation of neurospheres, and reduces the growth of organoids (fig. S2). These models mimic the first trimester of brain development, indicating that ZIKV infec-

tion during this developmental time window may result in severe damage. Other studies are necessary to further characterize the consequences of ZIKV infection during different stages of fetal development.

Cell death that impairs brain enlargement, calcification, and microcephaly are well described in congenital infections with TORCHS factors (3, 23, 24). Our results, together with recent studies showing brain calcification in microcephalic fetuses and newborns infected with ZIKV (10, 14), reinforce the growing body of evidence connecting the ZIKV outbreak to the increased reports of congenital brain malformations in Brazil.

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Editor's Summary

Zika virus tested in human brain organoids

The pernicious and resilient *Aedes* mosquito is rapidly spreading Zika virus (ZIKV) through the Americas. ZIKV infection mostly causes mild disease, but in some patients, nervous system involvement is indicated. A particular worry is an observed correlation between infection of mothers in the first trimester of pregnancy and microcephaly in newborns. Garcez *et al.* tested the effects of ZIKV compared with dengue virus infection on human neural stem cells grown as organoids. ZIKV targeted the human brain cells, reduced their size and viability in vitro, and caused programmed cell death responses.

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