Cite as: Garcez *et al.*, *Science* 10.1126/science.aaf6116 (2016).

Zika virus impairs growth in human neurospheres and brain organoids

Patricia P. Garcez,[2,1]* Erick Correia Loiola,[1]† Rodrigo Madeiro da Costa,[1]† Luiza M. Higa,[3]† Pablo Trindade,[1]† Rodrigo Delvecchio,[3] Juliana Minardi Nascimento,[1,4] Rodrigo Brindeiro,[3] Amilcar Tanuri,[3] Stevens K. Rehen[1,2]*

[1]D'Or Institute for Research and Education (IDOR), Rio de Janeiro, Brazil. [2]Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. [3]Institute of Biology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. [4]Institute of Biology, State University of Campinas, Campinas, Brazil.

*Corresponding author. Email: ppgarcez@icb.ufrj.br (P.P.G.); srehen@lance-ufrj.org (S.K.R.)

†These authors contributed equally to this work.

Since the emergence of Zika virus (ZIKV), reports of microcephaly have increased significantly in Brazil; however, causality between the viral epidemic and malformations in fetal brains needs further confirmation. Here, we examine the effects of ZIKV infection in human neural stem cells growing as neurospheres and brain organoids. Using immunocytochemistry and electron microscopy, we show 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, compromising 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, Syphilis) are the main congenital infections that compromise brain development *in utero* (*3*).

The 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, there is direct evidence that ZIKV is able to infect and cause death of neural stem cells (*15*).

Here, we used human induced pluripotent stem (iPS) cells cultured as neural stem cells (NSC), neurospheres and brain organoids to explore the consequences of ZIKV infection during neurogenesis and growth with 3D culture models. Human iPS-derived NSCs were exposed to ZIKV (MOI 0.25 to 0.0025). After 24 hours, ZIKV was detected in NSCs (Fig. 1, A to D), when viral envelope protein was shown 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 qRT-PCR (Fig. 1F), supporting 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, mock NSCs generated round neurospheres. However, ZIKV-infected NSCs generated neurospheres with morphological abnormalities and cell detachment (Fig. 2B). After 6 days in vitro (DIV), hundreds of neurospheres grew under mock conditions (Fig. 2, C and E). Strikingly, in ZIKV-infected NSCs (MOI 2.5 to 0.025) only a few neurospheres survived (Fig. 2, D and E).

Mock neurospheres presented expected ultrastructural morphology of nucleus and mitochondria (Fig. 3A). ZIKV-infected neurospheres revealed the presence of viral particles, similarly 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 (Fig. 3, B and F, arrows). Apoptotic nuclei, a hallmark of cell death, were observed in all ZIKV-infected neurospheres analyzed (Fig. 3B). Of note, ZIKV-infected cells in neurospheres presented smooth membrane structures (SMS) (Fig. 3, B and F), similarly to those previously described in 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 with ZIKV, and followed for 11 days in vitro (Fig. 4). The growth rate of 12 individual organoids (6 per condition) was measured during this period (Fig. 4, A and D). As a result of ZIKV infection, the average growth area of

ZIKV-exposed organoids was reduced by 40% when compared to brain organoids under mock conditions (0.624 mm^2 ± 0.064 ZIKV-exposed organoids versus 1.051 mm^2 ± 0.1084 mock-infected organoids normalized, Fig. 4E).

In addition to MOCK infection, we used dengue virus 2 (DENV2), a flavivirus with genetic similarities to ZIKV (*11*, *19*), as an additional control group. One day after viral exposure, DENV2 infected human NSCs with a similar rate as ZIKV (fig. S1, A and B). However, after 3 days in vitro, there was no increase in caspase 3/7 mediated cell death induced by DENV2 with the same 0.025 MOI adopted for ZIKV infection (fig. S1, C and D). On the other hand, ZIKV induced caspase 3/7 mediated cell death in NSCs, similarly to the results described by Tang and colleagues (*15*). After 6 days in vitro, there is a significant difference in cell viability between ZIKV-exposed NSCs compared to DENV2-exposed NSCs (fig. S1, E and F). In addition, neurospheres exposed to DENV2 display a round morphology such as uninfected neurospheres after 6 days in vitro (fig. S1G). Finally, there was no reduction of growth in brain organoids exposed to DENV2 for 11 days compared to MOCK (1.023 mm^2 ± 0.1308 DENV2-infected organoids versus 1.011 mm^2 ± 0.2471 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 to study embryonic brain development in vitro (*20*, *21*). While neurospheres present the very early characteristics of neurogenesis, brain organoids recapitulate the orchestrated cellular and molecular early events comparable 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 neural stem cells, disrupts the formation of neurospheres and reduces the growth of organoids (fig. S2), indicating that ZIKV infection in models that mimics the first trimester of brain development 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 impairing brain enlargement, calcification and microcephaly is well described in congenital infections with TORCHS (*3*, *23*, *24*). Our results, together with recent reports showing brain calcification in microcephalic fetuses and newborns infected with ZIKV (*10*, *14*) reinforce the growing body of evidence connecting congenital ZIKV outbreak to the increased number of reports of brain malformations in Brazil.

REFERENCES AND NOTES

1. E. C. Gilmore, C. A. Walsh, Genetic causes of microcephaly and lessons for neuronal development. *WIREs Dev. Biol.* **2**, 461–478 (2013). Medline doi:10.1002/wdev.89
2. C. G. Woods, J. Bond, W. Enard, Autosomal recessive primary microcephaly (MCPH): A review of clinical, molecular, and evolutionary findings. *Am. J. Hum. Genet.* **76**, 717–728 (2005). Medline doi:10.1086/429930
3. N. Neu, J. Duchon, P. Zachariah, TORCH infections. *Clin. Perinatol.* **42**, 77–103 (2015). Medline doi:10.1016/j.clp.2014.11.001
4. C. Zanluca, V. C. Melo, A. L. Mosimann, G. I. Santos, C. N. Santos, K. Luz, First report of autochthonous transmission of Zika virus in Brazil. *Mem. Inst. Oswaldo Cruz* **110**, 569–572 (2015). Medline doi:10.1590/0074-02760150192
5. G. S. Campos, A. C. Bandeira, S. I. Sardi, Zika virus outbreak, Bahia, Brazil. *Emerg. Infect. Dis.* **21**, 1885–1886 (2015). Medline doi:10.3201/eid2110.150847
6. E. B. Hayes, Zika virus outside Africa. *Emerg. Infect. Dis.* **15**, 1347–1350 (2009). Medline doi:10.3201/eid1509.090442
7. G. W. A. Dick, Zika virus. II. Pathogenicity and physical properties. *Trans. R. Soc. Trop. Med. Hyg.* **46**, 521–534 (1952). Medline doi:10.1016/0035-9203(52)90043-6
8. D. Musso, C. Roche, E. Robin, T. Nhan, A. Teissier, V. M. Cao-Lormeau, Potential sexual transmission of Zika virus. *Emerg. Infect. Dis.* **21**, 359–361 (2015). Medline doi:10.3201/eid2102.141363
9. B. D. Foy, K. C. Kobylinski, J. L. Chilson Foy, B. J. Blitvich, A. Travassos da Rosa, A. D. Haddow, R. S. Lanciotti, R. B. Tesh, Probable non-vector-borne transmission of Zika virus, Colorado, USA. *Emerg. Infect. Dis.* **17**, 880–882 (2011). Medline doi:10.3201/eid1705.101939
10. M. Sarno, G. A. Sacramento, R. Khouri, M. S. do Rosário, F. Costa, G. Archanjo, L. A. Santos, N. Nery Jr., N. Vasilakis, A. I. Ko, A. R. de Almeida, Zika virus infection and stillbirths: A case of hydrops fetalis, hydranencephaly and fetal demise. *PLOS Negl. Trop. Dis.* **10**, e0004517 (2016). Medline doi:10.1371/journal.pntd.0004517
11. G. Calvet, R. S. Aguiar, A. S. Melo, S. A. Sampaio, I. de Filippis, A. Fabri, E. S. Araujo, P. C. de Sequeira, M. C. de Mendonça, L. de Oliveira, D. A. Tschoeke, C. G. Schrago, F. L. Thompson, P. Brasil, F. B. Dos Santos, R. M. Nogueira, A. Tanuri, A. M. de Filippis, Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: A case study. *Lancet Infect. Dis.* 10.1016/S1473-3099(16)00095-5 (2016). Medline
12. A. S. Oliveira Melo, G. Malinger, R. Ximenes, P. O. Szejnfeld, S. Alves Sampaio, A. M. Bispo de Filippis, Zika virus intrauterine infection causes fetal brain abnormality and microcephaly: Tip of the iceberg? *Ultrasound Obstet. Gynecol.* **47**, 6–7 (2016). Medline doi:10.1002/uog.15831
13. R. B. Martines, J. Bhatnagar, M. K. Keating, L. Silva-Flannery, A. Muehlenbachs, J. Gary, C. Goldsmith, G. Hale, J. Ritter, D. Rollin, W. J. Shieh, K. G. Luz, A. M. Ramos, H. P. Davi, W. Kleber de Oliveria, R. Lanciotti, A. Lambert, S. Zaki, Notes from the field: Evidence of Zika virus infection in brain and placental tissues from two congenitally infected newborns and two fetal losses - Brazil, 2015. *MMWR Morb. Mortal. Wkly. Rep.* **65**, 159–160 (2016). Medline doi:10.15585/mmwr.mm6506e1
14. J. Mlakar, M. Korva, N. Tul, M. Popović, M. Poljšak-Prijatelj, J. Mraz, M. Kolenc, K. Resman Rus, T. Vesnaver Vipotnik, V. Fabjan Vodušek, A. Vizjak, J. Pižem, M. Petrovec, T. Avšič Županc, Zika virus associated with microcephaly. *N. Engl. J. Med.* **374**, 951–958 (2016). Medline doi:10.1056/NEJMoa1600651
15. H. Tang, C. Hammack, S. C. Ogden, Z. Wen, X. Qian, Y. Li, B. Yao, J. Shin, F. Zhang, E. M. Lee, K. M. Christian, R. A. Didier, P. Jin, H. Song, G. L. Ming, Zika virus infects human cortical neural progenitors and attenuates their growth. *Cell Stem Cell* **18**, 1–4 (2016). Medline
16. T. M. Bell, E. J. Field, H. K. Narang, Zika virus infection of the central nervous system of mice. *Arch. Gesamte Virusforsch.* **35**, 183–193 (1971). Medline doi:10.1007/BF01249709
17. C. Grief, R. Galler, L. M. C. Côrtes, O. M. Barth, Intracellular localisation of dengue-2 RNA in mosquito cell culture using electron microscopic in situ hybridisation. *Arch. Virol.* **142**, 2347–2357 (1997). Medline doi:10.1007/s007050050247
18. M. A. Lancaster, M. Renner, C. A. Martin, D. Wenzel, L. S. Bicknell, M. E. Hurles, T. Homfray, J. M. Penninger, A. P. Jackson, J. A. Knoblich, Cerebral organoids model human brain development and microcephaly. *Nature* **501**, 373–379 (2013). Medline doi:10.1038/nature12517
19. R. S. Lanciotti, O. L. Kosoy, J. J. Laven, J. O. Velez, A. J. Lambert, A. J. Johnson, S. M. Stanfield, M. R. Duffy, Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg. Infect. Dis.* **14**, 1232–1239 (2008). Medline doi:10.3201/eid1408.080287

20. B. A. Reynolds, S. Weiss, Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707–1710 (1992). Medline doi:10.1126/science.1553558
21. M. A. Lancaster, J. A. Knoblich, Generation of cerebral organoids from human pluripotent stem cells. *Nat. Protoc.* **9**, 2329–2340 (2014). Medline doi:10.1038/nprot.2014.158
22. J. G. Camp, F. Badsha, M. Florio, S. Kanton, T. Gerber, M. Wilsch-Bräuninger, E. Lewitus, A. Sykes, W. Hevers, M. Lancaster, J. A. Knoblich, R. Lachmann, S. Pääbo, W. B. Huttner, B. Treutlein, Human cerebral organoids recapitulate gene expression programs of fetal neocortex development. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 15672–15677 (2015). Medline
23. Z. W. Naing, G. M. Scott, A. Shand, S. T. Hamilton, W. J. van Zuylen, J. Basha, B. Hall, M. E. Craig, W. D. Rawlinson, Congenital cytomegalovirus infection in pregnancy: A review of prevalence, clinical features, diagnosis and prevention. *Aust. N. Z. J. Obstet. Gynaecol.* **56**, 9–18 (2016). Medline doi:10.1111/ajo.12408
24. D. V. Vasconcelos-Santos, D. O. Machado Azevedo, W. R. Campos, F. Oréfice, G. M. Queiroz-Andrade, E. V. Carellos, R. M. Castro Romanelli, J. N. Januário, L. M. Resende, O. A. Martins-Filho, A. C. de Aguiar Vasconcelos Carneiro, R. W. Almeida Vitor, W. T. Caiaffa, UFMG Congenital Toxoplasmosis Brazilian Group, Congenital toxoplasmosis in southeastern Brazil: Results of early ophthalmologic examination of a large cohort of neonates. *Ophthalmology* **116**, 2199–205.e1 (2009). Medline doi:10.1016/j.ophtha.2009.04.042
25. B. S. Paulsen, C. S. Souza, L. Chicaybam, M. H. Bonamino, M. Bahia, S. L. Costa, H. L. Borges, S. K. Rehen, Agathisflavone enhances retinoic acid-induced neurogenesis and its receptors α and β in pluripotent stem cells. *Stem Cells Dev.* **20**, 1711–1721 (2011). Medline doi:10.1089/scd.2010.0446
26. Y. Yan, S. Shin, B. S. Jha, Q. Liu, J. Sheng, F. Li, M. Zhan, J. Davis, K. Bharti, X. Zeng, M. Rao, N. Malik, M. C. Vemuri, Efficient and rapid derivation of primitive neural stem cells and generation of brain subtype neurons from human pluripotent stem cells. *Stem Cells Transl. Med.* **2**, 862–870 (2013). Medline doi:10.5966/sctm.2013-0080
27. E. A. Henchal, M. K. Gentry, J. M. McCown, W. E. Brandt, Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *Am. J. Trop. Med. Hyg.* **31**, 830–836 (1982). Medline

ACKNOWLEDGMENTS

The authors thank the lab-crew members Marcelo Costa, Ismael Gomes, Gabriela Vitória, Jarek Sochacki and Matías Alloati for providing technical support, cultures of human iPS cells and brain organoids. We acknowledge Dr. Ortrud Monika Barth for comments on the electron micrographs. We also thank Dr Fabricio Pamplona for Mind the Graph assistance and Centro Nacional de Biologia Estrutural e Bioimagem (CENABIO) for the use of the Electron Microscopy. Funds (not specifically for Zika virus studies) were provided by the Brazilian Development Bank (BNDES); Funding Authority for Studies and Projects (FINEP); National Council of Scientific and Technological Development (CNPq); Foundation for Research Support in the State of Rio de Janeiro (FAPERJ); and fellowships from the São Paulo Research Foundation (FAPESP, grant #2014/21035-0) and Coordination for the Improvement of Higher Education Personnel (CAPES). All protocols and procedures were approved by the institutional research ethics committee of Hospital Copa D'Or (CEPCOPADOR) under # 727.269. The authors declare no competing financial interests.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/science.aaf6116/DC1
Materials and Methods
Figs. S1 and S2
References (*25*–*27*)

2 March 2016; accepted 4 April 2016
Published online 14 April 2016
10.1126/science.aaf6116

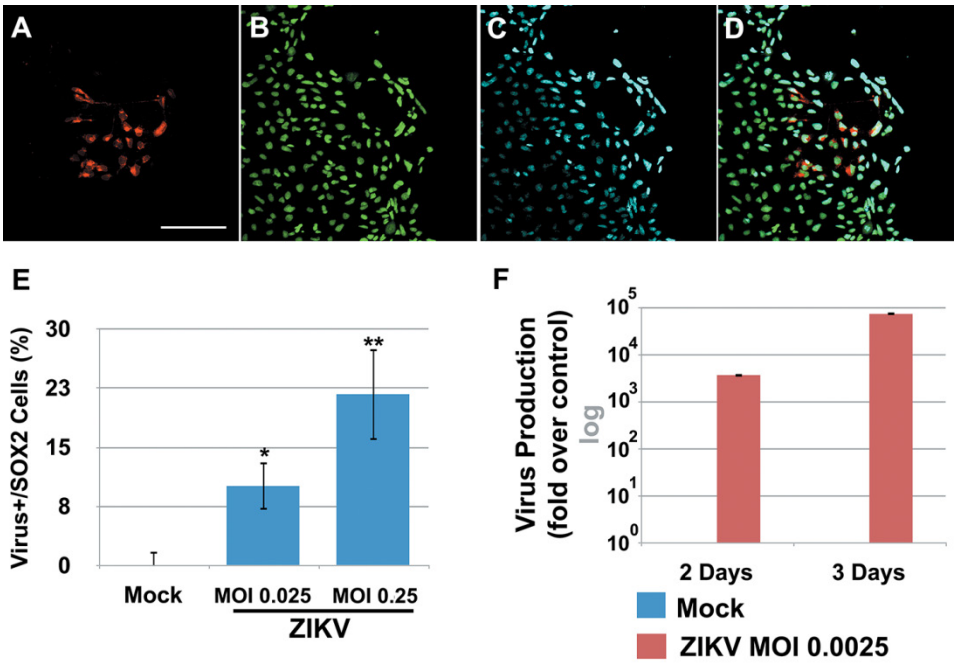

Fig. 1. ZIKV infects human neural stem cells. Confocal microscopy images of iPS-derived NSCs double stained for (**A**) ZIKV in the cytoplasm, and (**B**) SOX2 in nuclei, one day after virus infection. (**C**) DAPI staining, (**D**) merged channels show perinuclear localization of ZIKV. Bar = 100 µm. (**E**) Percentage of ZIKV infected SOX2 positive cells (MOI 0.25 and 0.025). (**F**) RT-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. RNA was extracted, qPCR performed and virus production normalized to 12h post-infection controls. Data presented as mean ± SEM, n=5, Student's *t* test, *$p < 0.05$, **$p < 0.01$.

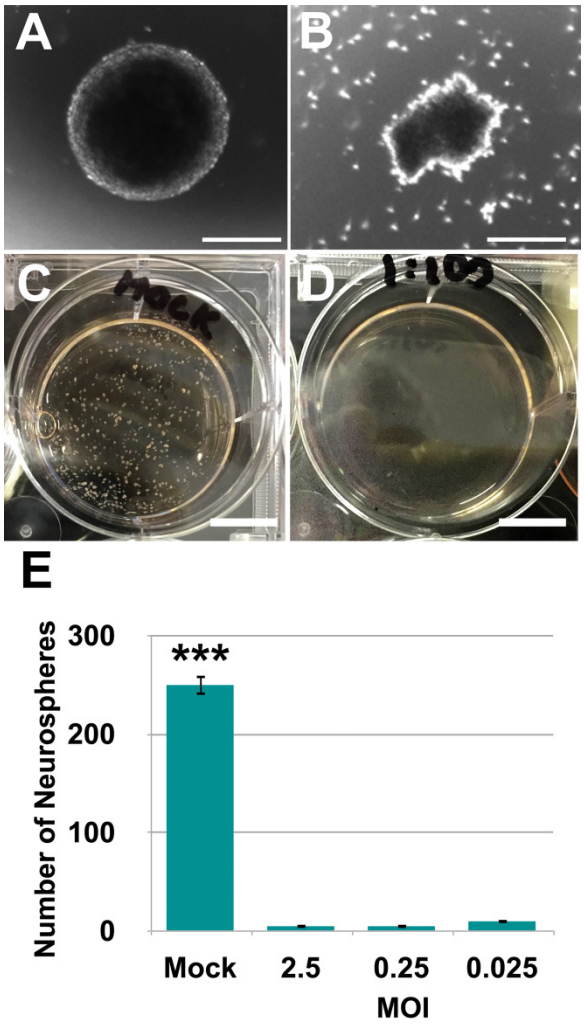

Fig. 2. ZIKV alters morphology and halts the growth of human neurospheres. (**A**) Control neurosphere displays spherical morphology after 3 DIV. (**B**) Infected neurosphere showed morphological abnormalities and cell detachment after 3 DIV. (**C**) Culture well-plate containing hundreds of mock neurospheres after 6 DIV. (**D**) ZIKV-infected well-plate (MOI 2.5-0.025) containing few neurospheres after 6 DIV. Bar = 250 µm in (A) and (B), and 1 cm in (C) and (D). (**E**) Quantification of the number of neurospheres in different MOI. Data presented as mean ± SEM, n=3, Student's *t* test, ***p < 0.01.

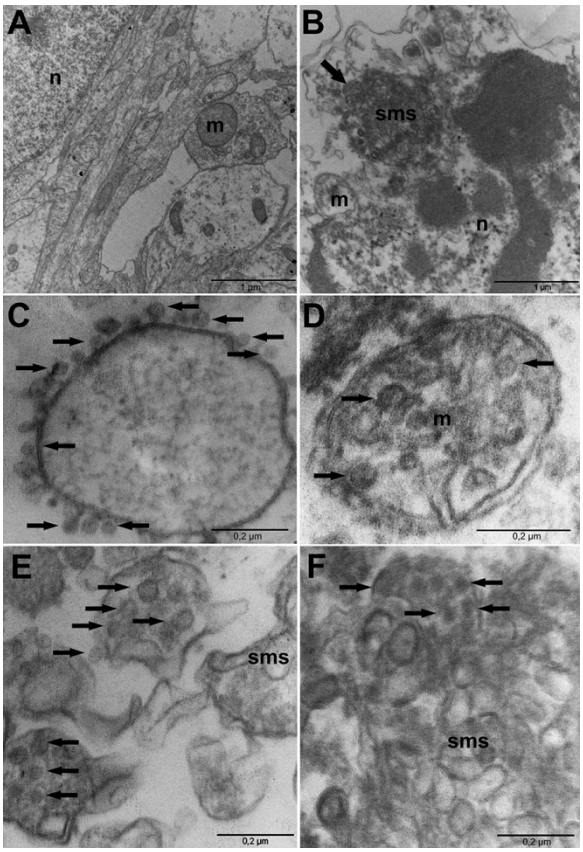

Fig. 3. ZIKV induces death in human neurospheres. Ultrastructure of mock- and ZIKV-infected neurospheres after 6 days in vitro. (**A**) Mock-infected neurosphere showing cell processes and organelles, (**B**) ZIKV-infected neurosphere shows pyknotic nucleus, swollen mitochondria, smooth membrane structures and viral envelopes (arrow). Arrows point viral envelopes on cell surface (**C**), inside mitochondria (**D**), endoplasmic reticulum (**E**) and close to smooth membrane structures (**F**). Bar = 1 µm in (A) and (B) and 0.2 µm in (C) to (F). m = mitochondria; n = nucleus; sms = smooth membrane structures.

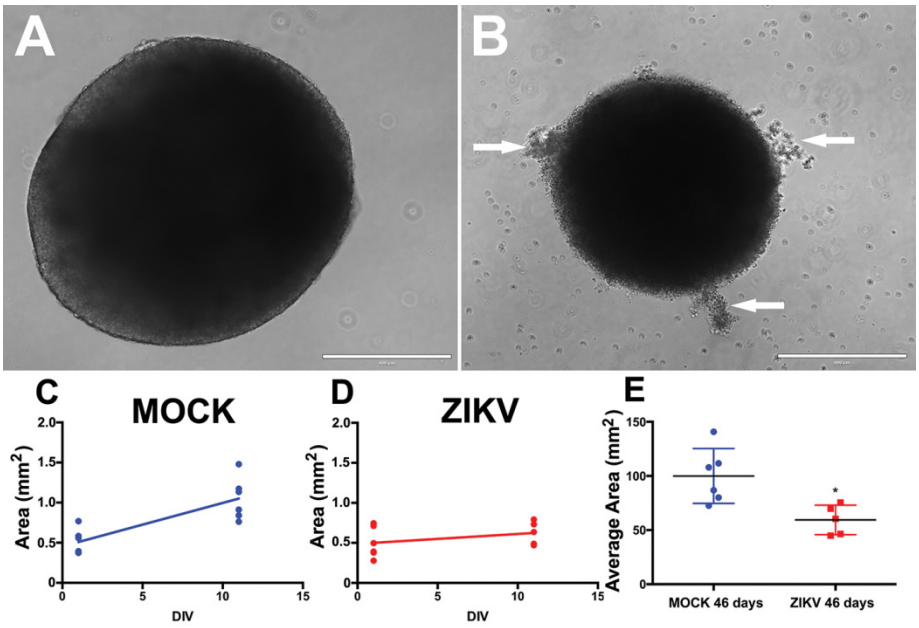

Fig. 4. ZIKV reduces the growth rate of human brain organoids. 35 days old brain organoids were infected with (**A**) MOCK and (**B**) ZIKV for 11 days in vitro. ZIKV-infected brain organoids show reduction in growth compared with MOCK. Arrows point to detached cells. Organoid area was measured before and after 11 days exposure with (**C**) MOCK and (**D**) ZIKV in vitro. Plotted quantification represent the growth rate. (**E**) Quantification of the average of mock- and ZIKV-infected organoid area 11 days after infection in vitro. Data presented as mean ± SEM, n=6, Student's *t* test, *p < 0.05.