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Zika virus and neural developmental defects: building a case for a cause

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Zika virus (ZIKV), a little-known *flavivirus* until a few months ago, is currently at the forefront of public health concerns worldwide because of its suspected role in causing microcephaly and other developmental defects in fetuses of infected mothers. On February 1, 2016, the World Health Organization declared a Public Health Emergency of International Concern (PHEIC) for ZIKV. Discovered more than half a century ago in Uganda, ZIKV remained endemic to parts of Africa and Asia until 2007 when an epidemic occurred in Micronesia, infecting approximately 5000 people. A larger outbreak occurred in French Polynesia in 2013, from which a suspected link to an increased rate of Guillain-Barré syndrome was reported. Most recently, the ongoing 2015 ZIKV outbreak in the Americas has so far seen the virus spreading to over 40 different countries and territories with increasing reports of fetus developmental defects and Guillain-Barré syndrome in areas affected by ZIKV. Two recent studies published on the same day, one clinical and one experimental, are critical first steps in building the case that ZIKV is the culprit behind these diseases.

ZIKV is a positive-stranded RNA virus and a member of the *flavivirus* genus in the family of *Flaviviridae*, which includes many other significant human pathogens such as dengue virus, West Nile virus, and Yellow fever virus. It was first isolated in 1947 from a rhesus monkey which was a subject of yellow fever research near Zika forest, Uganda. It was re-isolated in the *Aedes* mosquitoes in the same region soon after, suggesting the passage of the virus from monkey to insect vectors early on (Dick et al., 1952). The first human cases of ZIKV infection were reported in the late 1960s in east Africa and Southeast Asia. It is likely that the entire life cycle of ZIKV can be maintained between infected humans and biting mosquitoes without an intermediate animal host because one of the recent outbreaks occurred on an island without monkeys (Duffy et al., 2009). Several types of mosquitoes are known to be capable of transmitting ZIKV, including the ubiquitous *Aedes aegypti* and *Aedes albopictus*, both widely distributed throughout the world. Occasional sexual transmissions have also been reported, and vertical transmission from infected mother to their fetuses is likely (Figure 1) (Mlakar et al., 2016).

The genome of ZIKV is a RNA molecule of approximately 11kb in length, consisting of 5' and 3' untranslated regions (UTRs) flanking a single long open reading frame that encodes a polyprotein. Upon translation, the polyprotein is cleaved by host and viral proteases to produce individual viral proteins. The replication of ZIKV is through the action of the NS5 protein, a multi-functional enzyme that is partly a RNA-dependent RNA polymerase. The positive strand RNA of the virus is used as a template to make a negative strand RNA, which is then copied for the production of more positive strands to be packaged into the progeny virions. The replication and assembly processes of ZIKV occur on the intracellular membranes, which are likely amplified membrane structures of endoplasmic reticulum origin.

The sequence of the current strain of ZIKV causing the outbreak in the Americas closely matches that of the Asian lineage of ZIKV which had been circulating in Southeast

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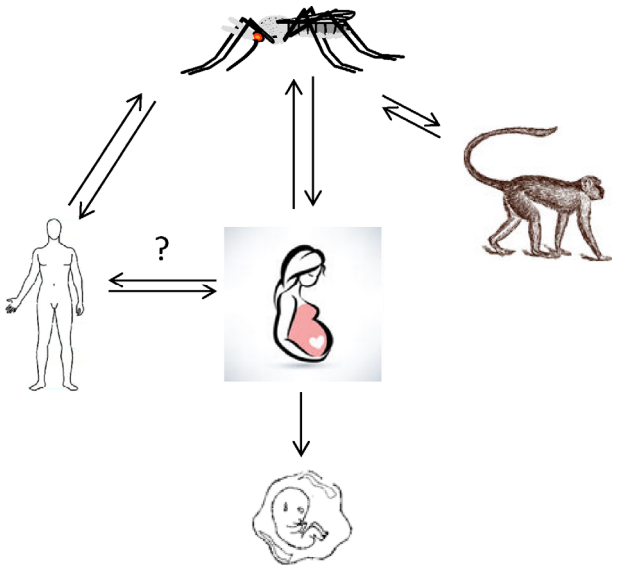

Figure 1 (color online) Possible transmission routes of ZIKV**.** After being isolated from a monkey, ZIKV was discovered in mosquitoes and then humans soon after. Sexual transmission is possible but rare. But infected pregnant women appear to have a high probability of transmitting the virus to their fetuses, leading to developmental defects including microcephaly.

Asia and the pacific islands (Enfissi et al., 2016). These sequences however, have diverged significantly from the original African strains (Haddow et al., 2012). Whether there are biological factors underlying the apparent lack of association between the African ZIKV strain and abnormal fetus development is not yet known.

The ZIKV epidemic in Brazil has resulted in over 1 million cases since March 2015. According to the report by the Brazil Health Ministry in October of 2015, this ZIKV outbreak coincides with an unusual increase in the number of the cases of neonates with microcephaly (insufficient brain development leading to a small head), which raised the first alarm about a potential link between ZIKV infection and microcephaly in babies born to an infected mother. Additionally, a recent report links the 2014–2015 French Polynesia ZIKV epidemic to Guillain-Barré Syndrome, another neurological disease (Cao-Lormeau et al., 2016). The virus appears to be spreading quickly since it reached the Americas in the current outbreak despite being essentially dormant and restricted to parts of Africa and Asian for many years. The prediction that ZIKV will likely cause outbreak in western countries, coupled with the social, ethical, and even religious implications of the "congenital Zika syndrome" has put this virus front and center in the public health forum. One of the most urgent questions is whether ZIKV infection is directly responsible for the increased cases of microcephaly and other neurological diseases.

As a first step to answer this question, our lab teamed up with the Song and Ming labs of Johns Hopkins University (JHU) to address if ZIKV can directly infect and impact human neural progenitor cells (hNPCs), which are responsible for cortical neuron differentiation in the fetus brain (Tang et al., 2016). The collaboration combines our experience in using stem cell differentiations as a model system for RNA virus infection (Wu et al., 2012) and the JHU labs' expertise in neurogenesis and brain development (Wen et al., 2014). Furthermore, the RNA-seq capability of the Jin lab at Emory University made it possible for us to obtain global gene expression changes induced by ZIKV in record time. We used embryonic cortical neural precursor cells derived from induced pluripotent stem cells (iPSCs) and found that ZIKV not only efficiently infected the hNPCs, but also led to the production of infectious ZIKV particles from the infected cells. At the same time, the infection efficiencies of the iPSCs and the differentiated immature neurons derived from the hNPCs were significantly lower. For example, starting with a MOI of 0.02, over 86% of the hNPCs were infected over a three day period, while the infected neurons under the same conditions were under 20%, similar to iPS cells. More importantly, ZIKV infection markedly perturbed the expression of genes related to cell cycle progression, which likely contributes to the increased apoptosis observed in the infected hNPCs. In addition to providing initial biological insights into ZIKV infection of brain cells, our study provides an entry point to study the long-term effect of ZIKV infection on brain development using a 3D organoïd model. Finally, we also generated a comprehensive dataset of differentially regulated genes in hNPCs upon ZIKV infection, a dataset which has been deposited online for all to analyze and examine (the accession number for RNA-seq data reported in this study is GEO: GSE78711).

In an independent study published in New England Journal of Medicine, Brasil et al. (2016) studied 88 pregnant women who had suspected symptoms of ZIKV infection, such as fever and rash within 5 days of their hospital visits in Rio de Janeiro. 72 of the women tested positive for ZIKV by RT-PCR in either serum, urine, or both, telling of the high prevalence of ZIKV infection in Brazil. Of the 42 ZIKV-positive women whom underwent fetal ultrasound, close to 30% (12/42) showed fetal abnormalities in contrast to 0% of ZIKV-negative women. Alarmingly, developmental defects in the fetus were not correlated with the timing of infection during pregnancy, as adverse results were found in mothers infected as late as over 30 weeks gestation. These results, coupled with a previous report of detecting ZIKV particles and antigen in fetal brain tissues of microcephaly cases (Mlakar et al., 2016), further strengthen the causal link between ZIKV and microcephaly.

The cell type used in our study is highly relevant for fetal brain development, particularly the region damaged in microcephaly patients. However, these experiments were performed in culture dishes and do not prove a causal link. In addition, due to accessibility issues early in the study, we used the African strain for our infection experiments. If strain-specific differences do exist, the different effect on

the developing fetus may not be at the cellular level or defined by the ability to affect hNPCs. The difference could be, for example, in the ability of the virus to evade clearance by the maternal immune system, to cross the placental barrier, or to generate specific cellular responses outside of the fetal brain tissue that underlie ZIKV pathogenesis. The clinical case reports so far contain a relatively small numbers of cases and animal model results have not yet been published for the Asian strain. Despite the limitations, these early studies represent valuable rapid responses from the scientific and clinical communities to an urgent global healthcare crisis, and add two very important pieces to the puzzle of ZIKV infection and microcephaly.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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