

Racing to Uncover the Link between Zika Virus and Microcephaly

Guo-li Ming, Hongjun Song, and Hengli Tang

In this Backstory, we give a behind-the-scenes account of the collaboration leading to our 2016 paper on Zika virus (ZIKV) infection of neural progenitor cells ([http://www.cell.com/cell-stem-cell/fulltext/S1934-5909\(16\)00106-5](http://www.cell.com/cell-stem-cell/fulltext/S1934-5909(16)00106-5)). It was one of the first clues into how ZIKV could be causing birth defects in babies from infected mothers.

The Problem

In 2015, a surge in cases of newborn brain defects that coincided with the outbreak of Zika virus (ZIKV) in Brazil captivated the world's attention. Researchers first discovered ZIKV in the blood of a rhesus macaque in the Ziika forest of Uganda in 1947 and re-isolated it from *Aedes* mosquitoes from the same geographic area a year later. Even though ZIKV had subsequently spread to the Asia-Pacific region, it only caused sporadic outbreaks and remained under the radar of clinicians, scientists, and the general public for over half a century until the recent outbreaks in South America. Historically, ZIKV had not been linked to any birth defect so scientists initially thought that the sudden rise in newborn brain defects in South America was related to environmental

Clockwise from top left: team members from Johns Hopkins (left to right: Kimberly M. Christian, Guo-li Ming, Hongjun Song, and Xuyu Qian), Florida State (Sarah Ogdén, Ruth Didier, Hengli Tang, Emily M. Lee, and Christy Hammack), and Emory (Feiran Zhang, Yujing Li, Peng Jin, and Zhexing Wen, a fellow from Guo-li's lab and now an assistant professor at Emory) and FSU's Sarah Ogdén working in the lab

factors. As the number of cases of both ZIKV infection and microcephaly grew, the suspicions that they were linked did as well. The World Health Organization (WHO) eventually declared a Public Health Emergency of International Concern (PHEIC) on February 1, 2016.

The brain defects came in the form of microcephaly, which is a condition in which a baby is born with an abnormally small head. This leads to long-lasting deficits in brain development and typically requires lifelong medical attention. Previous studies in the field had mostly focused on genetic causes of microcephaly, and a handful of risk genes had been identified. At the heart of the ZIKV crisis was the question of whether there is a causal link between ZIKV infection of pregnant mothers and microcephaly in their babies. We urgently needed the answer to this question, given its public health implications.

At the time, two of our collective laboratories led by Guo-li Ming and Hongjun Song at the Johns Hopkins University School of Medicine had been studying developmental brain disorders for over a decade. Using a miniaturized spinning bioreactor developed by three high school interns in our labs, we (Guo-li and Hongjun) had developed a human induced pluripotent stem cell (hiPSC)-based 3D brain organoid system by late 2015, which we thought could be a perfect experimental model system for addressing whether ZIKV causes microcephaly. The problem was that our labs had no access to and no experience working with ZIKV. In December 2015, we approached Wei Zheng, at the National Centers for Advancing Translational Sciences, who had previously worked on a drug screen for Ebola virus, but together we were not able to find a live ZIKV strain. Without a live strain, we determined that an alternative option would be to synthesize the cDNA for each of the encoded viral proteins and test their function in the organoid system. However, it would be entirely possible that (1) ZIKV does not affect brain development; or (2) ZIKV indirectly impacts the nervous system, such as via inflammation outside of the nervous system.

Meanwhile, the third group in our collaboration, headed by Hengli Tang at Florida State University, had been working on Hepatitis C viruses for the past decade, but we (the Tang lab) had just shifted our research focus to flaviviruses, specifically Dengue virus. In December 2015, we were geared up to produce a large batch of Dengue virus in a mosquito cell line when the news of ZIKV re-emergence broke, so we instead redirected several flasks of the mosquito cells to amplify the ZIKV virus. With the virus in hand, we were curious about the type of human cells ZIKV infects, as we recognized the importance of neural cells in this line of inquiry. However, we faced a significant hurdle: we had no experience with neural system analysis.

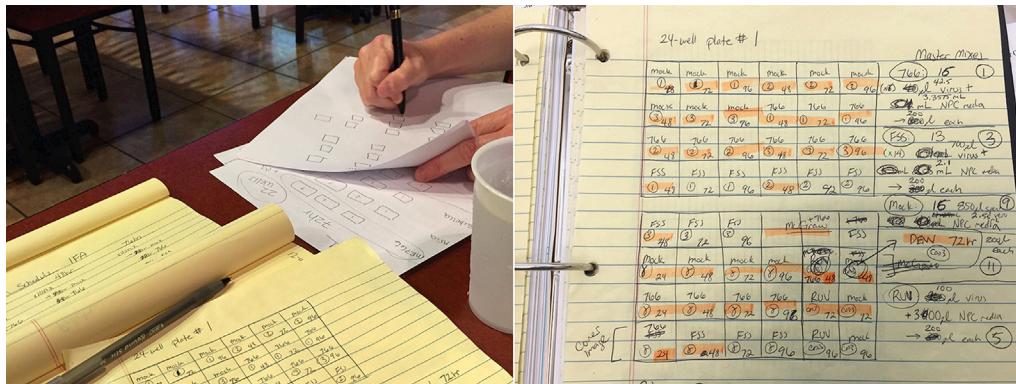
For a video introducing the lead researchers and the global health crisis that they addressed, visit the article online at [http://www.cell.com/cell-stem-cell/fulltext/S1934-5909\(17\)30175-3](http://www.cell.com/cell-stem-cell/fulltext/S1934-5909(17)30175-3).

Our Collaborative Approach

All three of us (Guo-li, Hongjun, and Hengli) felt an increasing sense of urgency when the WHO announced the PHEIC regarding ZIKV, microcephaly, and other neurological disorders on February 1, 2016. After this announcement, both of our teams in Maryland and our team in Florida became increasingly anxious to find the missing parts to complete a meaningful study. The three of us were in graduate school together in the Biological Sciences Program at University of California, San Diego and have since remained friends, spending some family vacations together. Over the years, we had discussed the idea of collaborating, but there just wasn't an opportunity to work together on a truly cohesive project. Hengli was planning to apply for an exploratory grant from the National Institutes of Health to support his new study on ZIKV and reached out to Hongjun for a collaboration letter on February 4, 2016. Within minutes of sending the email with "Zika" in the subject line, Hengli received a call from Hongjun. By the next day, our teams had ironed out a detailed plan to combine forces from three labs to ask two very simple, but very critical, questions: Does ZIKV directly infect neural cells during fetal brain development? If it does, what is the impact? We also decided to first perform a pilot experiment with ZIKV using 2D monolayer cultures to ask whether the virus infects cells in the neural lineage, followed by a 3D brain organoid study to test whether ZIKV induces a microcephaly-like phenotype.

For a video describing how the team discovered that ZIKV causes neural defects, visit the article online at [http://www.cell.com/cell-stem-cell/fulltext/S1934-5909\(17\)30175-3](http://www.cell.com/cell-stem-cell/fulltext/S1934-5909(17)30175-3).

At the heart of the ZIKV crisis was the question of whether there is a causal link between ZIKV infection of pregnant mothers and microcephaly in their babies. We urgently needed the answer to this question, given its public health implications.



Experimental planning to simultaneously test a large number of conditions

Because of the intricacies in handling human iPSCs for targeted neural differentiation in 2D and 3D culture conditions, as well as the lack of clarity about how to handle ZIKV safely, we couldn't simply send materials from one lab to another for all the experiments. We needed the personnel with the most experience on site to perform specific parts of the experiments. On the neurobiology side, Zhixing Wen, a research associate at the time in the Ming lab (now an assistant professor at Emory), had developed a very efficient approach to differentiate hiPSCs into a largely uniform population of human cortical neural progenitor cells (hNPCs), which can subsequently be differentiated into cortical neurons. Xuyu Qian, a biomedical engineering graduate student in the Song lab, together with a postdoctoral fellow in the Ming lab, Ha Nam Nguyen, had developed the 3D forebrain organoid system. On the virology side, two senior Ph.D. graduate students in the Tang lab, Christy Hammack and Sarah Ogden, had expertise in flavivirus propagation and amplification and were ready to apply their knowledge toward this timely and exciting new project. After making sure he created sufficient hiPSCs and hNPCs for shipment to the Tang lab, Zhixing booked his flight to FSU on February 8. The first batch of cells was shipped from JHU to FSU on February 9, and Zhixing arrived in Hengli's lab on February 10 to start the first set of experiments with Christy and Sarah.

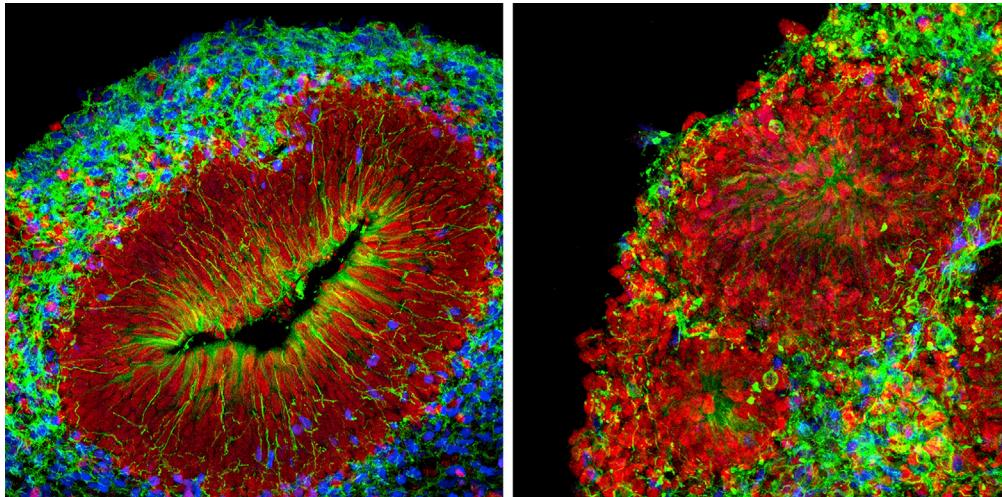
The “Ah Ha!” Moment

The first big moment of discovery came on Monday morning, February 15, 2016, when Sarah was looking through a fluorescence microscope at various cells exposed to ZIKV. She and Christy had come to the lab very early that morning to finish staining the cells with an antibody that recognizes the ZIKV envelope protein. Although hESCs and hiPSCs showed very little positivity for the ZIKV envelope antigen, they saw a substantially high infection rate for hNPCs under the same conditions. The staining showed the classic “virus factory” pattern for typical flaviviruses that assemble on ER-derived intracellular membranes. Importantly, the differentiated neurons from the same batch of hNPCs also showed a much lower susceptibility to the virus, suggesting a preferential targeting of hNPCs. As the Ming/Song labs had already shown that these hNPCs are forebrain-specific and representative of the cells responsible for generating cortex during early development, this singular result stood out as the first piece of experimental evidence for linking ZIKV infection to a brain-development defect. Hengli immediately relayed these exciting results to the JHU team and we all realized that this preferential infection of hNPCs, discovered in what were initially planned as a pilot experiment, would be of immediate interest to the research community and the public. Our teams followed up on these initial results and found that ZIKV induces cell cycle arrest, and causes growth defects and apoptosis of hNPCs, which further boosted the confidence of this link between ZIKV and brain developmental defects.

Because of the intricacies in handling human iPSCs ... [and] lack of clarity about how to handle ZIKV safely ... [w]e needed the personnel with the most experience on site to perform specific... experiments.

The 3 a.m. Morning Call

We didn't get a 3 a.m. call, exactly, but we did need to mobilize and recruit additional team members at a moment's notice. Once we saw the first results of hNPC infection and neural



Control organoid, left, and ZIKV-infected organoid, right

development deficits by ZIKV, we wanted to investigate the underlying molecular mechanism with transcriptome analysis. But how could we get RNA-seq done in a rapid time frame? We were lucky to catch Peng Jin, a longtime collaborator of the Song/Ming labs, on the ski slopes at Keystone, Colorado. He quickly arranged for his lab members and sequencing machine to be ready to receive samples and prepare for libraries, sequencing, and bioinformatics analysis at Emory University. In the end, all of the transcriptome analyses were completed in less than 4 days, instead of the weeks to months it normally takes.

The Publication Process

By February 16, 2016, after repeating the experiments again and seeing consistent results, we saw that we might have an important story to tell. Where should we submit the manuscript? Because of the global health emergency, Cell Press initiated a special policy for ZIKV-related manuscripts with an expedited reviewing process and limits on requiring additional experiments. We felt that our study could make a major impact on the scientific community by establishing a humanized model for ZIKV infection in the context of microcephaly, and that this model could be used to investigate underlying mechanisms and furthermore, to screen for drugs. Based on our exciting results in hNPCs, *Cell Stem Cell* appeared to be the perfect place for it! Hongjun had a phone conversation with the editor Christina Lillehook on February 18 and she immediately lined up three reviewers who agreed to review the manuscript. We submitted our manuscript at 12:30 a.m. on February 24. By 7:05 a.m. the same day, the manuscript was sent out for review, and reviewers' comments came back within 2 days. The reviewers were quite positive about our manuscript; however, they questioned the strength of our conclusion on the specificity of ZIKV infection for hNPCs compared to other cell types and requested some additional data and clarification. Luckily, we had accumulated additional data within this short reviewing period that could answer many of the questions. On the other hand, we did not have time to perform more controlled experiments to test the specificity of ZIKV infection among different cell types. We sent our response letter to Christina on Sunday morning, February 28. After a conference call with all PIs and Christina in the afternoon, along with multiple email exchanges among Christina, Debbie Sweet (the Editor-in-Chief of *Cell Stem Cell*), and us, we decided to drop our claim that ZIKV targets neural progenitors and reframed our conclusions to focus solely on our evidence that "ZIKV infects human neural progenitors." Thinking back, we were glad that we were conservative in making our conclusions based on solid evidence. Although many later studies from other independent groups demonstrated that our initial assertion was correct, including our own data from brain organoid experiments, the reviewers were right that we did not have sufficient evidence at the time. We submitted our revised manuscript during the night of February 28, and it was accepted the next day.

The success of this study was only possible with a seamless collaboration of groups with different and valuable expertise, as well as their perfect execution of a well-thought-out game plan.

Seamless Execution

Looking back, a key to the success of the project in such a short time frame was that our teams collectively planned all of the requisite experiments meticulously ahead of time and executed them in a parallel and staggered fashion. For example, we prepared several different cell types for testing, including different lines of pluripotent stem cells (hESCs and hiPSCs from different subjects), hNPCs, neurons, and other standard cell lines. To test a large number of conditions simultaneously, we scaled up both virus and cell production to meet the need. Also, to ensure that we could perform the proper number of independent experiments, Zhexing continuously generated and shipped hNPCs from JHU to FSU on a daily basis. The coordination of four labs at three universities at three different locations was seamless. We also had a lot of luck on our side as nearly all of the experiments worked better than we imagined, not just the first time, as happens so often, but also in multiple repeating experiments. Finally, there was no substitute for the time spent by all the members of the team. The virology team, Christy and Sarah, spent countless hours, both night and day, setting up the infections, collecting samples, and searching for images of infection into the wee hours of the morning. The hNPC expert, Zhexing, took round-trip flights within the same day just to make sure the hNPCs were seeded properly, and the RNA-seq team performed their tasks around the clock. Text messages were constant among fellows across different sites: What solution to use? When to fix the cells? When should we expect to receive the antibody shipment? We, the PIs, then spent evenings teleconferencing and revising drafts throughout the night.

Lessons Learned

Our collective work, which was published online on March 4, 2016 ([http://www.cell.com/cell-stem-cell/fulltext/S1934-5909\(16\)00106-5](http://www.cell.com/cell-stem-cell/fulltext/S1934-5909(16)00106-5)), received tremendous attention, both in the scientific field and from the general public. It was the leading headline out of all Google news for 6 hours in the afternoon, featured on the front page of the *New York Times* and *The Washington Post*, and highlighted on the NIH Director's blog a few weeks later. One crowning moment was on April 13, 2016, when the Center for Disease Control (CDC) announced, with an unprecedented speed, that ZIKV causes microcephaly. Our study, along with related basic and clinical findings, contributed to the CDC's decision to make this impactful announcement. Throughout all this excitement, we were pleased that our unbiased experimental approach, as well as careful interpretation of our data that avoided overstating our findings and conclusions, pleased both our scientific peers and the general public.

The success of this study was only possible with a seamless collaboration of groups with different and valuable expertise, as well as their perfect execution of a well-thought-out game plan. This initial success has led to an expansion of our collaborative research, with a number of new groups participating in series of follow-up studies, including demonstration of microcephaly-like deficits in human brain organoids, a drug repurposing screening with Wei Zheng at NCATs, animal studies with Zhiheng Xu at Institute of Genetics and Developmental Biology at Chinese Academy of Sciences, and studies of other flaviviruses, such as Dengue virus and West Nile virus, with Margo Ann Brinton at Georgia State University. Meanwhile, many scientists around the world have joined the effort to address the impact of ZIKV on the nervous system. Findings similar to ours have been independently reported and the field continues to advance at hyper-speed because the consequences of ZIKV infections can be so dire and the potential benefits of the research are so clear.