The Journal of Infectious Diseases

BRIEF REPORT

Fetal Growth Restriction Caused by Sexual Transmission of Zika Virus in Mice

Ryuta Uraki,[1,a] Kellie Ann Jurado,[2,a] Jesse Hwang,[1] Klara Szigeti-Buck,[3] Tamas L. Horvath,[3] Akiko Iwasaki,[2,4] and Erol Fikrig[1,4]

[1]Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, [2]Department of Immunobiology, and [3]Program in Integrative Cell Signaling and Neurobiology of Metabolism, Department of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut; and [4]Howard Hughes Medical Institute, Chevy Chase, Maryland

Zika virus (ZIKV) can be transmitted by mosquito bite or sexual contact. Using mice that lack the type I interferon receptor, we examined sexual transmission of ZIKV. Electron microscopy analyses showed association of virions with developing sperm within testes as well as with mature sperm within epididymis. When ZIKV-infected male mice were mated with naive female mice, the weight of fetuses at embryonic day 18.5 was significantly reduced compared with the control group. Additionally, we found ocular deformities in a minority of the fetuses. These results suggest that ZIKV causes fetal abnormalities after female mating with an infected male.

Keywords. Zika virus; sexual transmission; fetal growth abnormalities; electron microscopy.

Zika virus (ZIKV), a mosquito–borne flavivirus, is an emerging pathogen that causes fever, rash, joint pain, and conjunctivitis. Although ZIKV was initially thought to result in mild symptoms, recent epidemics have been associated with more severe presentations, including Guillain-Barre syndrome, congenital defects in newborns, and poor pregnancy outcomes [1]. Zika virus is spread through the bite of an infected mosquito, and several medical case reports have indicated sexual transmission. In fact, 2% (n = 10/482 cases) of ZIKV disease cases reported by residents of the United States have been determined to be transmitted by sexual contact (Centers for Disease Control and Prevention, unpublished data), including a case study where ZIKV was transmitted from a man with no symptoms to a ZIKV-naive woman [2]. Previously, we have shown that vaginal ZIKV infection of pregnant female mice at various gestational time points led to fetal growth restriction and infection of the fetal brain [3]. We, and other groups, have shown that a ZIKV infection of male mice causes testicular damage due to persistent virus replication within this organ [4–6]. In addition, consistent with human case reports [7, 8], infectious viral particles are found within semen for prolonged periods of time [4–6]. These reports also support ZIKV sexual transmission. In this study, we investigated testicular shedding dynamics of ZIKV and the effect of sexual transmission of ZIKV from infected male mice to naive female mice and the impact on the fetus.

Received 27 February 2017; editorial decision 17 April 2017; accepted 27 April 2017; published online May 2, 2017.

[a]R. U. and K. A. J. contributed equally to this study.

Correspondence: E. Fikrig, MD, Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, Anlyan Center for Medical Research and Education, 300 Cedar St, New Haven, CT 06520 (erol.fikrig@yale.edu).

The Journal of Infectious Diseases® 2017;215:1720–4

DOI: 10.1093/infdis/jix204

METHODS

Cells and Virus

C6/36 *Aedes albopictus* cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 1% tryptose phosphate broth at 30°C in 5% carbon dioxide (CO_2). Vero cells (African green monkey kidney epithelial cells; ATCC) were maintained in DMEM with 10% FCS at 37°C in 5% CO_2. These cells have been routinely confirmed to be mycoplasma free. An Americas-derived ZIKV (MEX2-81 strain; referred to as $ZIKV^{MEX}$) isolated in 2016 was obtained from the University of Texas Medical Branch at Galveston's World Reference Center for Emerging Viruses and Arboviruses. To generate the virus stock, C6/36 cells were infected with ZIKV and incubated up to 10 days. Cell-free supernatants were collected, and Vero cells were used for plaque assays.

Plaque Assays

Serial-diluted ZIKV were incubated for 1 hour at 37°C in 5% CO_2. Then, Vero cells seeded in 12-well plates were overlaid with 2% agarose and 2× media. Four days after infection, cells were fixed by 4% paraformaldehyde/phosphate-buffered saline (PBS) and stained with 0.005% amido black, and plaques were counted to calculate plaque forming unit (PFUs).

Mouse Experiments

Ifnar1$^{-/-}$ mice (C57BL/6 background) aged 6–8 weeks were analyzed in this study. Mice were bred in a specific-pathogen-free facility at Yale University. Mice were inoculated with ZIKV by subcutaneous (volume of 50 µL, 10^5 PFUs) or intravaginal (volume of 10 µL, 750 PFUs) routes [3]. For vaginal infection during diestrus phase, virgin female mice were treated with medroxyprogesterone acetate (also known as Depo-Provera; Pfizer) 5 days before infection. To access the sexual transmission, *Ifnar1*$^{-/-}$ male mice infected with 10^5 PFUs of $ZIKV^{MEX}$ subcutaneously were cohoused with naive *Ifnar1*$^{-/-}$ female mice at 3 days after infection. Pregnancy models were timed by the presence of a plug, indicating gestational age embryonic day (E) 0.5. Female mice were separated from male mice after a plug. At E18.5, blood of pregnant mice and brains of fetuses were harvested to detect antibody responses or the virus, respectively.

To assess virus growth in testes and epididymis, 3 mice were euthanized at 5 days after infection, and their organs were collected, homogenized with DMEM with 10% FCS, and titrated.

Detection of Virus-Specific Antibodies

Virus-specific antibodies (immunoglobulin G) in serum from mice infected with ZIKV or mice cohoused with naive female mice were measured by using an enzyme-linked immunosorbent assay (ELISA). The ELISA plates were coated overnight at 4°C with recombinant ZIKV envelope protein (Mybiosource, MBS5304721). After being blocked with 2% skim milk for 1 hour at room temperature, the plates were then incubated with test samples serially diluted in PBS for 1 hour at room temperature. The plates were washed by PBS with 0.05% Tween 20 (PBS-T), then incubated with an horseradish peroxidase-conjugated antimouse immunoglobulin G antibody. After the plates were washed again with PBS-T, 3,3',5,5'-tetramethylbenzidine solution was added and incubated for 15 minutes at room temperature. The reaction was stopped by the addition of 1M sulfuric acid. The optical density at 450 nm (OD_{450}) was measured.

Electron Microscopy

The tissues were fixed in 2.5% formaldehyde/glutaraldehyde in phosphate buffer. Samples were secondarily fixed with osmium tetroxide, negative stained with uranyl acetate, treated in ascending alcohols, and embedded in Durcupan ACM (EMS 14040). Seventy-nanometer ultrathin sections were cut on a Leica ultramicrotome, and the images were obtained on FEI transmission electron microscopy (TEM).

Statistics

Data analysis was performed using GraphPad Prism. A 2-tailed Student's *t* test was used to determine significance of body weight of E18.5 pups. A 1-way analysis of variance followed by Tukey's multiple comparison test was used to determine the significance of antibody responses.

Study Approval

This study was performed in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

RESULTS

To further elucidate testicular shedding dynamics of ZIKV, we used TEM to visualize virions within the testes (developing sperm) and in epididymal homogenate (mature sperm). We found virus particles surrounding developing sperm at the luminal edge of the seminiferous epithelium of the testes as they are being released by the Sertoli cells during the process of spermination (Figure 1A). Transmission electron microscopy of sperm within epididymal homogenate demonstrated ZIKV particles to be attached to mature sperm (Figure 1B). We further found virions to be present within cytoplasmic droplets attached to mature sperm (Figure 1B) and associated with epdidymal residual bodies (Figure 1B). These observations are consistent with high virus titer in testes and epididymis at 5 days after infection (Figure 1C). We clearly observed a trend where ZIKV-infected mice had a higher proportion of sperm with cytoplasmic droplets compared with epididymal homogenate from mock-infected mice.

Because we and others have shown that *Ifnar1*$^{-/-}$ mice are susceptible to ZIKV through intravaginal exposure [3, 9], we aimed to develop a mouse model of sexual transmission by mating an infected *Ifnar1*$^{-/-}$ male mouse with a naive *Ifnar1*$^{-/-}$ female mouse. To this end, male *Ifnar1*$^{-/-}$ mice were infected subcutaneously with 10^5 PFUs of ZIKV^{MEX}. As we have previous shown a high level of viremia to be detected 3–7 days after infection [6], we cohoused naive *Ifnar1*$^{-/-}$ female mice with infected male mice at 3 days after infection. Female mice were monitored for the presence of a copulation plug and dissected 18 days after plug detection (Figure 2A). Fetal weight was significantly reduced in pups from female mice cohoused with infected male mice compared with pups from the control female mice cohoused with naive male mice (Figure 2B). Of note, some fetuses displayed clear evidence of intrauterine growth restriction (n = 4/38 fetuses from 5 pregnant mice) as compared with those from the control *Ifnar1*$^{-/-}$ mice at E18.5 (Figure 2B). Strikingly, we also observed fetuses with ocular malformations in 2 (from different litters) of 38 fetuses from mating with the infected sire. We also detected virions in fetus brain by TEM (Figure 2C). These results strongly demonstrate that ZIKV can transmit from an infected male to a naive female and causes fetal birth abnormalities.

To determine whether sexual transmission of ZIKV resulted in measurable viral replication in the vaginal mucosa, we examined ZIKV RNA levels in the vaginal wash in the dams every other day after sexual contact. However, we could not detect the ZIKV RNA in the vaginal wash, suggesting that ZIKV replicated minimally within the genital tract of the dams. To assess whether naive females seroconverted and produce ZIKV-specific antibodies, we examined antibody responses against ZIKV envelope protein by ELISA. As shown in Figure 2D, 1 of 7 pregnant females generated ZIKV-specific antibodies over the time points examined, suggesting that ZIKV transmitted from infected male *Ifnar1*$^{-/-}$ mouse to female *Ifnar1*$^{-/-}$ mouse. Because high virus titers were detected in testes and epididymis in infected male *Ifnar1*$^{-/-}$ mice at 5 days after infection (Figure 1C), we estimated virus titers in the inoculated semen and infected female mice intravaginally to be approximately 750 PFUs of ZIKV per ejaculate. As previous models of vaginal infection required the use of Depo-Provera, a progestin that drives female mice into the diestrus phase when the vaginal wall is the thinnest and most susceptible to viral infections [3, 9], we

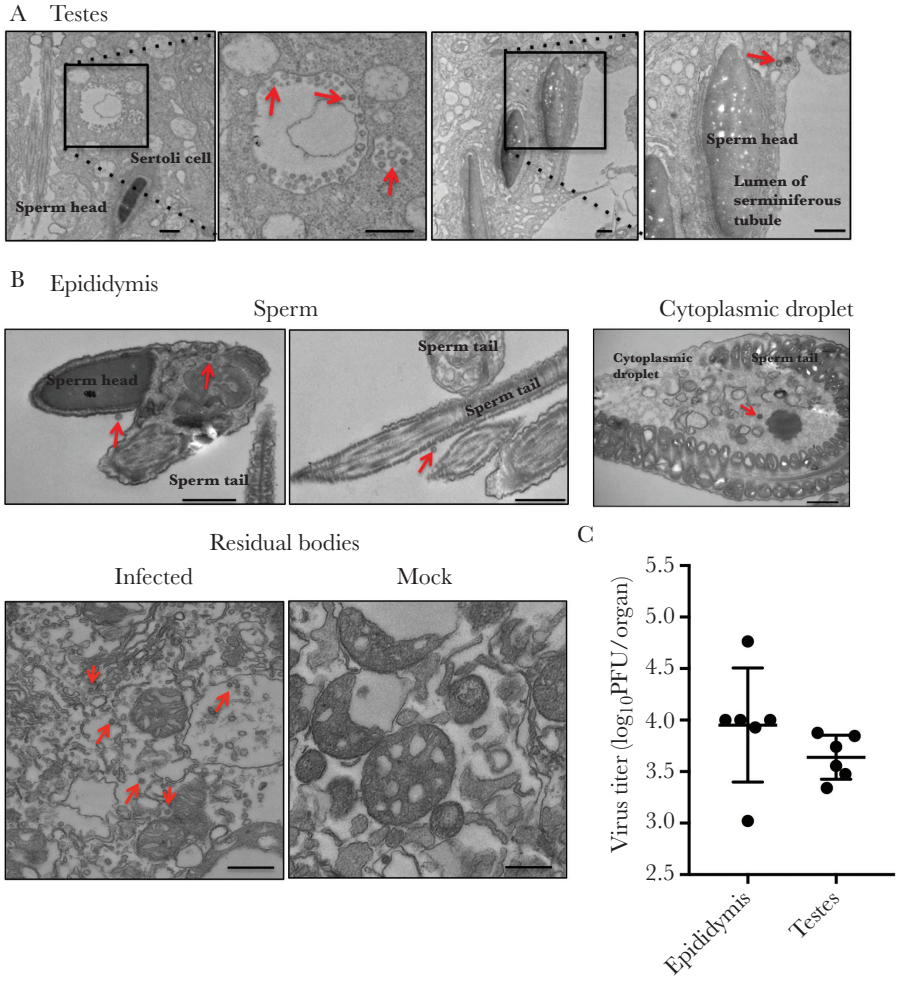

Figure 1. Virions were detected in testes and epididymis after Zika virus (ZIKV) infection. Transmission electron microscopy depicts ZIKV-infected cells in the testes (*A*) and epididymis (*B*) of *Ifnar1*$^{-/-}$ mice at 5 days after infection. Arrows indicate ZIKV virions within the infected cells. Scale bars = 500 nm. *C*, The ZIKV titers in testes and epididymis at 5 days after infection were examined by plaque assays using Vero cells. The results are expressed as the mean titers. The detection limit of plaque assays is 1.8 log_{10} plaque-forming units per gram.

examined the virus-specific responses with or without Depo-Provera treatment. Although direct intravaginal infection with 750 PFUs of ZIKV without the use of Depo-Provera did not induce virus-specific antibody in the serum at 21 days after infection, mice treated with Depo-Provera elicited significantly higher virus-specific antibodies (Figure 2D).

DISCUSSION

Sexual transmission of arthropod-borne viruses before ZIKV has not previously been documented. Yet, there have been several incidences where a confirmed ZIKV infection has been found among sexual partners where arthropod involvement in transmission was not suspected [10]. These reports suggest ZIKV is unique among flaviviruses in this transmission ability.

We now demonstrate using a mouse model that ZIKV can be sexually transmitted and cause intrauterine growth restriction in fetus/pups. Similar to Ebola virus, ZIKV is able to persist in semen for a long time [11], and our TEM results show intact virions both on and within sperm, suggesting that seminal fluid, as well as the sperm within the semen, can serve as a vehicle of transmission. This finding suggests the possibility that ZIKV could invade an ovum with a sperm, causing direct infection of the ovum, resulting in deleterious developmental effects. Semen also contains proteins and lipids. A previous study showed that certain seminal factors, such as semen-derived amyloid fibrils, facilitate virion attachment to cells and increase the infectivity of human immunodeficiency virus type 1 in vitro [12]. Although the contribution of seminal fluid on ZIKV infection has not yet been investigated, seminal factors that enhance or suppress ZIKV infection could potentially be examined using our sexual transmission model. Further, we noted that ZIKV-infected mice had a higher proportion of sperm with cytoplasmic droplets compared with mock-infected mice. The retention of cytoplasmic droplets on sperm has been associated with infertility. These observations, therefore, may help to explain why sperm from ZIKV-infected mice exhibit reduced fertility [13]. Although ZIKV RNA and antigens have been previously detected

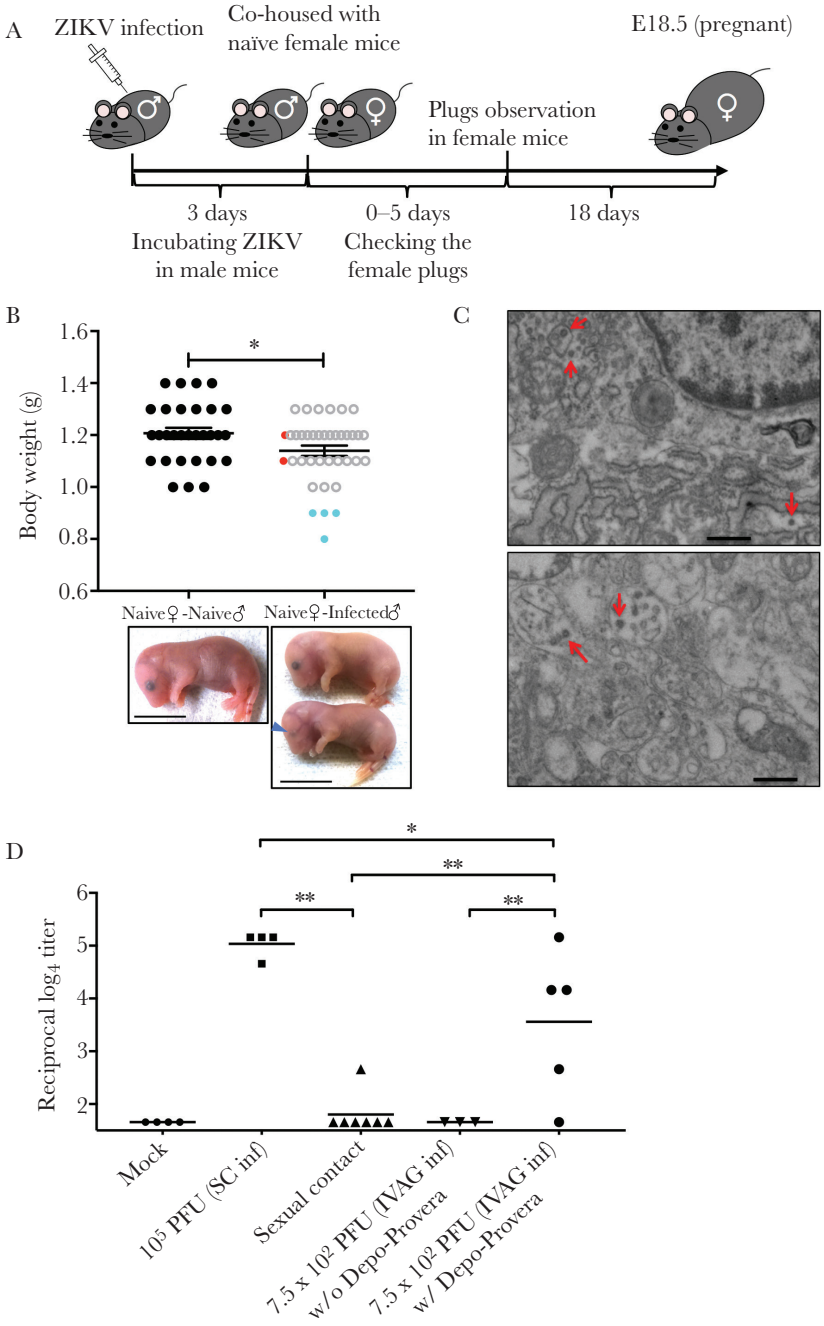

Figure 2. Zika virus (ZIKV) transmits from infected males to females and causes fetal growth restriction. *A*, Schematic of the experimental model for sexual transmission is described. *B*, Total body weight in pups at embryonic day 18.5 (E18.5) from infected animals (n = 30 pups from 4 separate litters cohousing with uninfected male mice; n = 38 pups from 5 separate litters cohousing with infected male mice; Student's *t* test, $*P < .05$, average ± SEM). Pups with red or blue symbols show the ocular malformation or growth restriction, respectively. Pictures represent the typical *Ifnar1–/–* pups at E18.5. Blue arrow shows ocular malformations in the pups. Scale bar = 1 cm. *C*, Zika virus–infected cells of fetal brain from the female cohousing with the infected male killed on E18.5 were observed by transmission electron microscopy. Arrows indicate the ZIKV virions within the infected cells. Scale bar = 500 nm. *D*, Zika virus envelope protein–specific antibodies in serum were measured using an enzyme-linked immunosorbent assay. The detection limit of reciprocal titer is 1.7. $*P < .05$ and $**P < .01$ by 1-way analysis of variance followed by Tukey's multiple comparison test. Abbreviations: inf, infected; IVAG, intervaginally; PFU, plaque-forming unit; SC, subcutaneously; ZIKV, Zika virus.

in sperm [5, 8], our TEM data definitively demonstrates that sperm are ZIKV carriers and strongly support sexual transmission.

Our study also demonstrates that female mice can acquire ZIKV through the vaginal route during the peak of estrus phase, a stage required for successful pregnancy. Interestingly we found direct intravaginal infection with 750 PFUs of ZIKV without the use of Depo-Provera did not induce virus-specific antibody production, but the administration of Depo-Provera elicited significantly higher virus-specific antibodies. These results suggest that vaginal ZIKV inoculation

from males by semen may be insufficient to induce an antibody response against ZIKV during the peak of estrus phase, a stage required for successful pregnancy. Interestingly, 1 of 2 female mice cohoused with an infected male mouse for 3 weeks, presumably resulting in multiple mating events, showed seroconversion without pregnancy (data not shown), suggesting that viral input from multiple seminal inoculations or viral input during diestrus phase can elicit adaptive immunity. Nevertheless, our results are consistent with the notion that the vaginal route of infection with ZIKV is more efficient during the diestrus-like phase than the estrus-like phase [14].

Recently a ZIKV sexual transmission model was published using female CD-1 or AG129 mice mated with ZIKV-infected male AG129 mice, whereas we used the type I interferon receptor–deficient mouse [15]. Although this group demonstrated sexual transmission, here we add to the literature through finding fetal growth restriction and ocular malformations. In conclusion, we demonstrated that ZIKV is associated with sperm, transmits from male to female sexually, and causes fetal abnormalities. This sexual transmission model recapitulates phenotypes observed in humans and may be useful for drug or vaccine evaluation in vivo.

Notes

Acknowledgements. R. U., K. A. J., A. I., and E. F. conceived the study. R. U., K. A. J., A. I., and E. F. designed the experiments. R. U., K. A. J., and J. H. performed the experiments and analyzed the data. K. S. and T. L. H provided intellectual guidance in the production and interpretation of TEM data. R. U., K. A. J., A. I., and E. F. wrote the manuscript with contributions from all coauthors. We thank Sarah Householder and Laura J. Yockey (Yale University) for discussion.

Financial support. This work was supported by the National Institutes of Health (5T32 AI007019-40 to K. A. J.; 4T32HL007974-15 to J. H.; and R21AI131284 to A. I.). E. F. and A. I. are investigators within the Howard Hughes Medical Institute.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Ventura CV, Maia M, Bravo-Filho V, Góis AL, Belfort R Jr. Zika virus in Brazil and macular atrophy in a child with microcephaly. Lancet **2016**; 387:228.
2. D'Ortenzio E, Matheron S, Yazdanpanah Y, et al. Evidence of sexual transmission of Zika virus. N Engl J Med **2016**; 374:2195–8.
3. Yockey LJ, Varela L, Rakib T, et al. Vaginal exposure to Zika virus during pregnancy leads to fetal brain infection. Cell **2016**; 166:1247–56 e4.
4. Ma W, Li S, Ma S, et al. Zika Virus causes testis damage and leads to male infertility in mice. Cell **2016**; 167:1511–24 e10.
5. Govero J, Esakky P, Scheaffer SM, et al. Zika virus infection damages the testes in mice. Nature **2016**; 540:438–42.
6. Uraki R, Hwang J, Jurado KA, et al. Zika virus causes testicular atrophy. Sci Adv **2017**; 3:e1602899.
7. Nicastri E, Castilletti C, Liuzzi G, Iannetta M, Capobianchi MR, Ippolito G. Persistent detection of Zika virus RNA in semen for six months after symptom onset in a traveller returning from Haiti to Italy, February 2016. Euro Surveill **2016**; 21:doi: 10.2807/1560-7917.ES.2016.21.32.30314.
8. Mansuy JM, Suberbielle E, Chapuy-Regaud S, et al. Zika virus in semen and spermatozoa. Lancet Infect Dis **2016**; 16:1106–7.
9. Khan S, Woodruff EM, Trapecar M, et al. Dampened antiviral immunity to intravaginal exposure to RNA viral pathogens allows enhanced viral replication. J Exp Med **2016**; 213:2913–29.
10. Hills SL, Russell K, Hennessey M, et al. Transmission of Zika virus through sexual contact with travelers to areas of ongoing transmission—continental United States, 2016. MMWR Morb Mortal Wkly Rep **2016**; 65:215–6.
11. Purpura LJ, Choi MJ, Rollin PE. Zika virus in semen: lessons from Ebola. Lancet Infect Dis **2016**; 16:1107–8.
12. Münch J, Rücker E, Ständker L, et al. Semen-derived amyloid fibrils drastically enhance HIV infection. Cell **2007**; 131:1059–71.
13. Cooper TG. Cytoplasmic droplets: the good, the bad or just confusing? Hum Reprod **2005**; 20:9–11.
14. Tang WW, Young MP, Mamidi A, Regla-Nava JA, Kim K, Shresta S. A mouse model of Zika virus sexual transmission and vaginal viral replication. Cell Rep **2016**; 17:3091–8.
15. Duggal NK, Ritter JM, Pestorius SE, et al. Frequent Zika virus sexual transmission and prolonged viral RNA Shedding in an immunodeficient mouse model. Cell Rep **2017**; 18:1751–60.