Potential mechanisms of Zika-linked microcephaly

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A recent outbreak of Zika virus (ZIKV) in Brazil is associated with microcephaly in infants born of infected mothers. As this pandemic spreads, rapid scientific investigation is shedding new light on how prenatal infection with ZIKV causes microcephaly. In this analysis we provide an overview of both microcephaly and ZIKV, explore the connection between prenatal ZIKV infection and microcephaly, and highlight recent insights into how prenatal ZIKV infection depletes the pool of neural progenitors in the developing brain. © 2017 The Authors. *WIREs Developmental Biology* published by Wiley Periodicals, Inc.

MICROCEPHALY, BRAIN GROWTH, AND DEVELOPMENT

Microcephaly is a disease that can arise from defects in brain development or from degenerative events following normal brain development. Microcephaly and microencephaly (small brain) are often used synonymously. Microcephaly is characterized by an extremely small head and associated with intellectual disability. Microcephalic individuals typically exhibit head circumferences that are more than two standard deviations below the mean of age-matched controls with dramatic reductions in size of the cerebral cortex.[1–4] Primary microcephaly, the focus of this primer, describes a group of brain development diseases in which fewer neurons are produced and the resulting smaller brains generally maintain neurotypical gyral patterns.[2,5] Primary microcephaly may arise from a variety of genetic and environmental changes, including maternal viral infections.[1,2,5] Insights into the cellular and molecular mechanisms governing proliferation of neuronal progenitors and their subsequent differentiation into neurons provide the foundation for understanding the etiology of microcephaly.

The most dramatic loss of neurons in all types of microcephaly occurs in the cerebral cortex, a multilayered collection of neurons and glia in the forebrain that comprises nearly 80% of total brain mass in humans.[6] Although all mammals have a cortex, the human cortex is dramatically expanded, and without a fully formed cortex, higher-order processes such as those required for cognition and sensation are impaired.[7] The cortex, like the rest of the brain, originates from the pseudostratified epithelium of the neural tube. In the developing neural tube, progenitor cells initially stretch between the apical (ventricular) and basal (pial) surfaces. These cells undergo three possible modes of division: symmetric proliferative divisions that produce two progenitors, asymmetric divisions that produce one progenitor and one neuron, and symmetric differentiating divisions that produce two neurons. A balance between symmetric proliferative divisions and asymmetric divisions dominates early development. Later, the cell cycle lengthens and progenitors are more likely to undergo symmetric differentiating divisions. Together with programmed cell death, the relative proportions of these modes of division are precisely controlled, ultimately determining the final number of neurons. Early changes in the balance of asymmetric and symmetric divisions impact central nervous system (CNS) development, resulting in pathologies that arise from either too many or too few neurons (reviewed in Ref 8). These basic principles apply to development of all regions of the brain, including the cortex.

During the earliest stages of forebrain development, neuroepithelial cells lining the ventricles divide

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symmetrically, expanding the pool of neural stem cells (NSCs) that will ultimately generate all neurons and glia. As development proceeds and the cortex begins to emerge, these neuroepithelial cells divide asymmetrically, establishing a pool of NSCs and beginning the production of postmitotic neurons. The NSCs that give rise to the majority of neurons in the cerebral cortex are radial glia (RG). RG are specialized cells that share hallmarks of both glia and neuronal progenitor cells.[9–13] As neuroepithelial cells and RG proliferate, their nuclei migrate along the apicobasal axis through a process termed interkinetic nuclear migration (INM). The nuclear position along the apicobasal axis provides a read-out for cell cycle progression, with mitotic nuclei positioned apically and G1/S nuclei basally.[14] Studies of developing neuroepithelia, primarily in the zebrafish retina (e.g., Refs 15–17), and also in the mammalian cortex (e.g., Refs 18,19), suggest that the kinetics of INM and nuclear dwell time near the basal surface may influence whether progenitors produce differentiating daughter cells.

During corticogenesis, which begins around eight weeks postconception in humans, NSCs divide asymmetrically.[13,20] In primates, NSCs are found in two distinct proliferative regions—the ventricular zone (VZ) and the outer subventricular zone (SVZ;). In the VZ, each RG extends a short apical process toward the ventricular surface and a long basally directed process toward the pial surface, providing a migratory track for newly-born neuronal progeny.[21] In the SVZ, transit amplifying non-RG cells (also known as intermediate progenitors) reside in the inner SVZ while RG-like cells, termed outer RG (oRG), occupy the outer SVZ.[22] Despite retaining only a basal process and no apical process, oRG divide asymmetrically, producing another proliferating oRG progenitor and a differentiating neuron. As they progress through the cell cycle, oRG exhibit mitotic soma translocation, a process somewhat similar to INM,[23,24] that is essential for balancing proliferation and differentiation in the outer SVZ. Once established, the SVZ expands dramatically, especially in humans, and supports extended and extensive cortical neurogenesis.[12,23] Over the next 7 weeks of development, RG and oRG divide asymmetrically and transit amplifying cells divide symmetrically, together generating postmitotic progeny that differentiate into nearly 13 billion excitatory neurons which comprise approximately 80% of all neurons in the cortex (reviewed in Refs 13,20). The other approximately 20% of neurons in the cortex are inhibitory local circuit neurons that arrive in their final location by migrating radially from the VZ and SVZ or tangentially from the ganglionic eminence of the ventral forebrain.[25,26] By 22 weeks postconception, nearly all RG and oRG have undergone differentiating divisions and cortical neurogenesis slows to undetectable levels, but continued gliogenesis, myelination, and dendrite dynamics support brain growth for years after birth.[1,20] Deviations in these patterns of cell behaviors that support neurogenesis, especially those that negatively influence renewing divisions of RG and oRG, can lead to microcephaly (for example, see Figure 1a).

CELLULAR AND MOLECULAR MECHANISMS OF MICROCEPHALY

Although many features of neural progenitor behavior are conserved among mammals, the human cerebral cortex contains gene expression patterns and cell populations (e.g., oRG) that enable extended proliferation and neurogenesis during development (reviewed in Ref 20). Modeling microcephaly in traditional animal models, therefore, poses a number of challenges. Nonetheless, studies in mice with alterations in genetic loci that have been linked to microcephaly in humans reveal that elevated levels of neuronal and/or neuronal progenitor apoptosis as well as precocious differentiation of neuronal progenitors underlie a number of cortical defects, including microcephaly (reviewed in Refs 20,27,28). In addition, recent advances in induced pluripotent stem cell (iPSC) and embryonic stem cell (ESC) technologies enable analyses of neural cells and tissues grown *in vitro* (e.g., Ref 29). These three-dimensional culture protocols can generate neurospheres and cerebral organoids that mimic neural growth and development,[30] providing a powerful complement to *in vivo* mouse studies.

Human genetic studies have identified a clear link between mutations in 12 different genes and primary microcephaly (see Table 1). Many of these gene products are enriched in neuronal progenitor cells and impact mitotic cell behaviors that center on the centrosome (e.g., chromosome segregation) and DNA repair (see Table 1 and references therein). Mutations in genes that regulate passage through M-phase and/or promote differentiation likely underlie many cases of primary microcephaly. *Microcephalin* (*MCPH1*) is important for passage through the G2/M checkpoint, influencing both centrosome and DNA repair functions.[43] Another microcephaly-linked gene, *CENPJ/CPAP*, is important for centrosome integrity and duplication.[44,45] Disruption of this gene is linked to randomization of cell division

TABLE 1 | Genes Disrupted by Both Zika Virus and Microcephaly

Gene	Proposed Gene Product Function
CDK5RAP2 (CDK5 regulatory subunit-associated protein 2)	Centrosome protein, localizes to centrosome during mitosis[31]
MCPH1 (MICROCEPHALIN)	G2/M checkpoint regulator[32]
CASC5 (cancer susceptibility candidate 5)	Formation of kinetochore-microtubule attachments, chromosome segregation[33]
WDR62 (WD repeat-containing protein 62)	Regulation of brain development, localizes to the nucleus and centrosome during mitosis[34]
ASPM (abnormal spindle-like microcephaly-associated protein)	Regulation of mitotic spindle in neuroprogenitor cells[35]
CENPJ/CPAP (centromere protein J)	Regulation of centrosome integrity and spindle morphology[36]
STIL (SCL/TAL1-interrupting locus)	Regulation of mitotic spindle checkpoint[37]
CEP135 (centrosome protein of 135 kDa)	Maintains structure and organization of the centrosome[38]
CEP152 (centrosome protein of 152 kDa)	Localizes to centrioles, involved in cell progression through mitosis[39]
ZNF335 (zinc finger protein 335)	Controls neural progenitor self-renewal, neurogenesis, and neuronal differentiation[40]
PHC1 (polyhomeotic-like protein 1)	Involved in chromatin remodeling/cell cycle regulation[41]
CDK6 (cyclin-depenent kinase 6)[a]	Associates with centrosome during mitosis and regulates centrosome number[42]

[a] The only microcephaly-linked gene with expression unchanged by ZIKV infection of animals or cerebral organoids.

planes and decreased number of proliferating progenitor cells.[36,46] Another centrosome gene product, *CDK5RAP2*, is also linked to microcephaly, with decreased/loss of *CDK5RAP2* function associated with reduced numbers of neural progenitor cells (NPCs) caused by a switch from symmetric proliferative divisions to asymmetric divisions, most likely by randomizing the cleavage plane.[31,36] Indeed, cerebral organoids made from human cells with truncated *CDK5RAP2* were smaller, contained fewer progenitors, and exhibited disruptions cell polarity.[29] At least 5 of the 12 genes linked to primary microcephaly are required for centrosome activity, suggesting that the developing brain has a more pronounced need for centrosome function than other tissues.

Environmental factors, especially vertical transmission of viruses from mother to child, have also been associated with microcephaly. Prenatal infection with cytomegalovirus (CMV), herpes simplex (HSV), and rubella have been shown to cause microcephaly and other neurological disorders, with CMV contributing the majority of noninherited cases of primary microcephaly.[47] Recent high-profile examples also link prenatal Zika virus (ZIKV) infection to primary microcephaly.[48–50] Recent surveys of gene expression reveal that 11 microcephaly-linked genes are downregulated in tissues from ZIKV-associated microcephaly.[51,52] All of these genes are cell cycle regulated and because ZIKV perturbs cell cycle progression, it is likely decreased expression of these genes is an indirect effect of ZIKV infection.[52,53] Nonetheless, these data raise the possibility that prenatal ZIKV infection may trigger many of the same cellular defects associated with primary microcephaly (Table 1).

ZIKV OVERVIEW

ZIKV is a flavivirus endemic to Africa and Asia. It contains a positive-strand, RNA genome that encodes a single long open reading frame that is posttranslationally modified to generate structural and nonstructural ZIKV proteins.[54–56] ZIKV can and has evolved rapidly, with distinct strains isolated from numerous locations throughout the world (Refs 57,58; see also Box 1). Structural studies of ZIKV indicate that its protein coat, comprised primarily of envelope (E) protein, is similar to other flaviviruses.[59,60] There may be ZIKV-specific and/or strain-specific changes associated with ten polymorphic amino acids around an asparagine-linked carbohydrate moiety in the E protein, as this region appears to be subject to selective pressure upon multiple passages in insect cell lines.[60,61]

Traditionally, ZIKV was thought to be a mild vector-borne disease that is transmitted by mosquitoes, primarily *Aedes aegypti* or *Aedes albopictus* (both of which are found throughout tropical and subtropical climates, including the contiguous USA). ZIKV can also be sexually transmitted, and high viral titers have been found in semen.[50,62,63] Animal studies suggest that ZIKV may also be transmitted via

ocular secretions including tears.[64] ZIKV appears to target cells with an inherent immune privilege such as NPCs,[65] retinal cells,[66] and testicular cells[67]; its compact, stable structure enables its survival in relatively harsh physiological conditions including semen, saliva, and urine.[59,63,68,69] Most relevant to this primer, a number of recent studies have isolated ZIKV from amniotic fluid, placentae, and tissues of microcephalic infants[48,50,70–73] illustrating that ZIKV can pass from the maternal blood stream into the developing CNS in her fetus. For additional details about ZIKV detection and diagnosis, see Box 2.

HOW DOES ZIKV ENTER CELLS?

Despite its initial isolation in the mid-20th century, the mechanisms of ZIKV infection are poorly understood. How ZIKV enters human cells is an active area of investigation. Like other flaviviruses, ZIKV infects cells when it interacts with cell surface receptors and is then internalized via endocytosis. Potential candidate receptors for ZIKV on human cells include receptor tyrosine kinases (RTKs) in the TYRO, AXL, and MER (TAM) family. Members of the TAM family typically interact with ligands that bind phosphotidylserine, a lipid found predominantly on the surfaces of apoptotic cells and enveloped viruses. The TAM RTKs were originally thought to only regulate homeostasis of mature tissues,[74,75] but additional evidence shows that TAM receptors are also found on proliferating progenitor cells in developing neural tissues.[76,77] For example, one member of the TAM family, AXL, is expressed on proliferating neuroepithelial cells in the brain and retina but not on mature neurons. AXL receptors appear to be enriched in neuroepithelial VZ areas in mice, ferrets, and human cerebral organoids,[77] and may serve as one entry route for a number of flaviviruses.[78] Recent studies have demonstrated that depletion of AXL reduces ZIKV infection of cultured fibroblasts[79] and astrocytes.[80] Although these data raise the possibility that AXL may provide a route for ZIKV entry, recent work shows that ZIKV infection rate and cell viability were the same in iPSC-derived neural progenitors and cerebral organoids with or without AXL.[81] Moreover, depletion of AXL in an immunodeficient mouse model did not inhibit eye or brain infection by ZIKV.[82] Alternative candidate ZIKV entry receptors include other TAM RTKs—TYRO3 and MER—as well as other receptors known to facilitate entry of enveloped viruses such as those in the T-cell and immunoglobulin and mucin domain family (e.g., TIM1). These receptors can promote infection by other flaviviruses including Dengue (DENV) and West Nile Virus (WNV)[78,83] and have been found to facilitate ZIKV infection in cultured cells (e.g., Refs 79,81,84). Together, these data suggest that Zika virions likely use multiple methods for entry dependent on cell type and context of infection.

Current evidence demonstrates that ZIKV induces microcephaly in humans when passed vertically from mother to fetus, particularly when the mother is infected during the first trimester of pregnancy. The mechanism by which ZIKV attaches to and crosses the placenta remains unclear, and the likelihood of viral infection may change based on placental age. One study exposed a variety of human placental cells to Zika virions, finding that placental trophoblastic cells (PTCs) from full-term placentae were resistant to ZIKV infection, protected by the type III interferon they constitutively secrete. However, placental cell lines were sensitive to ZIKV infection, but were protected by conditioned media from noninfected PTCs. ZIKV infection of primary placental cells and chorionic villus explants is strongly linked to the enveloped virus receptor, TIM-1.[85] Consistent with this finding, administration of duramycin, a compound that prevents binding between TIM1 protein and enveloped viruses, was highly effective in decreasing ZIKV infection in cell culture.[84] Whether levels of TIM1 decrease as type III interferon secretion increases in PTCs has not yet been determined.

HOW DOES ZIKV CAUSE MICROCEPHALY?

Reports from Brazil in 2016 first correlated maternal ZIKV infection with an increased likelihood of congenital defects, especially microcephaly. It has since become clear that ZIKV exhibits tropism for NPCs and that prenatal infection with this virus induces cell cycle arrest, apoptosis, and differentiation defects in the developing nervous system.[51,53,86,87] To cross from the maternal blood stream into the developing fetal brain, ZIKV must cross the placental barrier and the developing blood–brain barrier (BBB). Studies that model the BBB in culture (hBMEC) or investigate ZIKV infection in adult mice suggest that ZIKV can infect BBB cells and cross the mature BBB, but it remains unclear exactly how.[87,88]

Primary microcephaly is established during the first trimester of pregnancy when cortical

A BRIEF HISTORY OF ZIKV STRAINS AND INFECTIONS

ZIKV strains can be grouped into two major groups based on sequence similarities—African and Asian.[89] ZIKV was first isolated from a rhesus monkey in 1947 in the Zika forest in Uganda, and human cases of ZIKV were documented there and in other areas of East Africa beginning in the 1950s.[57] By the mid-1950s, ZIKV was found scattered throughout East and West Africa, India, and Southeast Asia. A unique strain isolated in Malaysia in 1966 is the most likely parent of currently circulating, microcephaly-linked Asian strains.[57] The first large-scale human infections were associated with Asian strains in Micronesia and French Polynesia.[90–92] In March 2015, ZIKV was detected in the Americas and reached epidemic levels in Brazil in December 2015, with upwards of 1.3 million reported cases.[50] The strains circulating in Brazil are most closely related to an Asian strain isolated in 2013 in French Polynesia. Multiple ZIKV isolates, especially those of the Asian group, exhibit evidence of rapid mutation, including adaptive changes for human infection.[57,89,93] Analyses of 50 currently circulating Asian strains (isolated from Micronesia as well as South and Central America) exhibit conserved variation when compared to African strains, especially within structural proteins and this may be linked to increased ZIKV virulence over the past 10 years.[89,94,95] For additional information about the diverging and myriad ZIKV strains, see also Refs 96–98.

neurogenesis is most pronounced. The same appears to be true for ZIKV-linked microcephaly, with the most severe cases of ZIKV-associated microcephaly correlating with maternal infection during the first trimester.[99,100] Embryonic and fetal neural progenitors in the developing forebrain appear to be especially susceptible to adverse effects of ZIKV infection. A number of reports correlate maternal ZIKV infection with microcephaly as well as developmental ocular abnormalities.[50,101–103] Unlike related flaviviruses, such as WNV and DENV, which can infect neural cells but either target mature neurons (WNV) or elicit a less cytotoxic response (DENV), ZIKV exhibits clear tropism for proliferative neural cells, often with cytotoxic effects.[53,104] ZIKV also productively and detrimentally infects cranial neural crest cells (CNCC), which give rise to cranial bones and can exert paracrine effects on the developing brain.[105]

Once Zika virions enter neurogenic regions in the brain, they reduce the number of mitotic progenitor cells by promoting cell cycle arrest and apoptotic cell death,[51,52,86,87,106,107] autophagy,[55,88] or possibly by precocious differentiation (Figure 1b). ZIKV is not the only flavivirus that triggers apoptosis and autophagy. For example, both DENV and WNV can trigger caspase activation to induce apoptosis.[108,109] In addition, a number of flaviviruses have been shown to increase autophagy, presumably in an effort to evade the host immune system and furthering their own replication.[110–113] Thus, ZIKV appears to cause microcephaly not because it elicits ZIKV-specific responses but because it triggers cell behaviors that perturb proliferation and survival of NPCs during critical periods of brain development. ZIKV may also nonautonomously promote microcephaly by promoting precocious cell cycle exit and differentiation of cortical progenitors, as infection of CNCC causes secretion of proteins that promote neuronal differentiation.[105]

ZIKV infection after the first trimester can also detrimentally impact neural development. Some infants, who were not classified as microcephalic at birth and who may have been infected during the second or even third trimester of development, have been shown to develop microcephaly and other neurological disorders. In fact, of 12 infants diagnosed with microcephaly within months after birth, only three showed prenatal ultrasound abnormalities consistent with ZIKV infection.[114] In addition, ZIKV infection has been associated with an increased likelihood of Guillain–Barré Syndrome,[115,116] an autoimmune disorder that targets the peripheral nervous system in children and adults.

INSIGHTS FROM MODELING ZIKV-LINKED MICROCEPHALY

Given the neurotropic tendency of the virus and the particularly devastating effects of embryonic and early fetal ZIKV infection, *in vitro* studies examining NPCs are particularly necessary. To determine whether and how prenatal ZIKV infection causes microcephaly, researchers have employed a number of cell culture and animal models. Neural cell culture experiments have provided insight into ZIKV infection, and recently yielded an extensive list of genes dysregulated upon ZIKV infection. A study comparing the transcriptomes of human neural progenitors
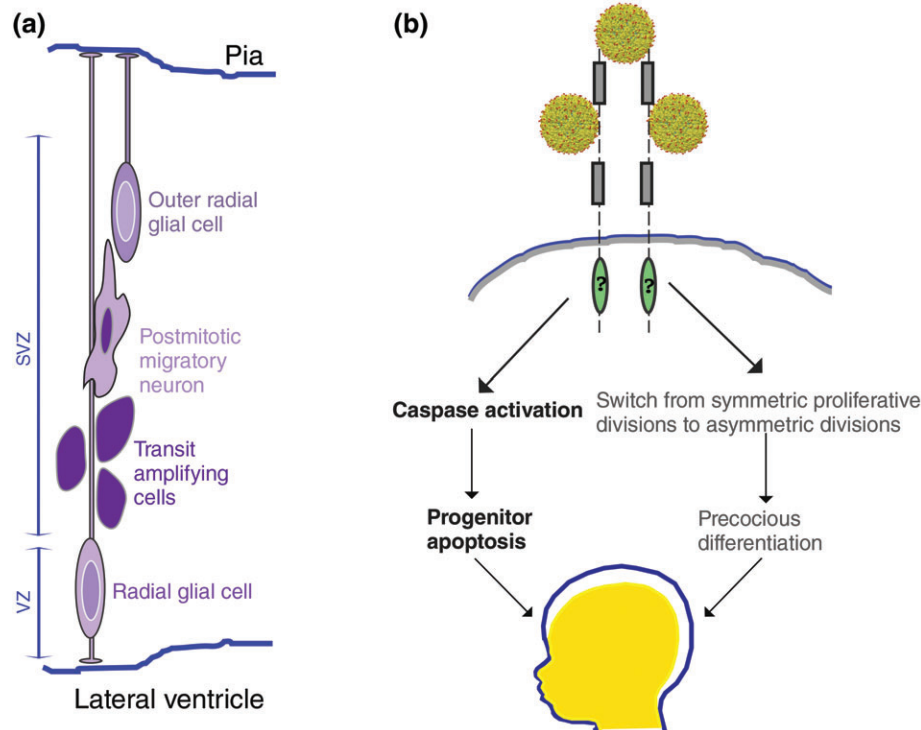
FIGURE 1 | (a) The human cortex develops and grows primarily from two populations of neural stem cells—radial glia [RG; light purple soma with dark purple nucleus positioned near the ventricle in the ventricular zone (VZ)] and outer radial glia [oRG; dark purple soma with light purple nucleus more basally located in the subventricular zone (SVZ)]. These cell types undergo self-renewing asymmetric divisions, giving rise to either transit amplifying (intermediate progenitor) cells (dark purple) or postmitotic migratory neurons. (b) ZIKV preferentially targets proliferating neural cells including RG, oRG, and neural crest cells. It is likely that a combination of viral-triggered apoptosis (left arrows) and precocious differentiation (right arrows) contribute to ZIKV-liked microcephaly. Candidate receptors for Zika virions entry include a number of phosphotidylserine receptors (e.g., AXL, Mer, Tyro3, and TIM1) and are symbolized with a basic domain structure including Ig-repeats (gray boxes) and kinase domains (green ovals). (Reprinted with permission from 60. Copyright 2016 The American Association for the Advancement of Science)

infected with a microcephaly-associated Asian ZIKV strain with other closely related flaviviruses (an African ZIKV and DENV) highlights the importance of apoptotic regulators and viral response genes.[121] For instance, the tumor suppressor and pro-apoptotic gene, *tp53* is elevated only in NPCs infected with the microcephaly-associated strain. Infection with this same strain of ZIKV also alters expression of immune system genes, including elevation of the Toll-like receptor 3 (*TLR3*) and its targets. TLR3 is present in neurons and NPCs and has been shown to modulate proliferation of NPCs and promote apoptosis (e.g., Refs 122,123).

A number of studies using three-dimensional culture protocols to generate neurospheres and cerebral organoids have yielded important insight into the unique pathogenicity of ZIKV. For example, forebrain mini-organoids were infected with ZIKV and observed at 4 and 14 days postinfection (dpi) to simulate prenatal ZIKV infection during the first trimester.[124] At 4 dpi, a negligible number of neurons were

infected but many neural progenitors showed signs of active ZIKV infection. By 14 dpi, many more cells were infected, indicative of a productive viral infection. The 14-dpi forebrain organoids contained fewer proliferating cells and increased numbers of apoptotic progenitors and neurons. ZIKV infection of older forebrain organoids, resembling second trimester fetal brains with oRG in the SVZ, showed ZIKV-positive RG and oRG, consistent with ZIKV tropism for NSCs.[124] To examine the early consequences of ZIKV infection, another study exposed NSCs, neurospheres, and cerebral organoids to ZIKV or a closely related flavivirus, DENV.[107] Both viruses were detected in NSCs 24 hours postinfection, but only ZIKV-containing cells exhibited hallmarks of apoptosis. Neurospheres cultured from ZIKV-infected NSCs failed to thrive, containing numerous Zika virions and apoptotic cell bodies. Similarly, brain organoids derived from human iPSCs and then exposed to ZIKV manifested signs of defective growth and neurogenesis, contained the remnants of apoptotic cells,

BOX 2

ZIKV DETECTION AND DIAGNOSIS

Because ZIKV contains a positive-strand RNA genome, which can simply be translated by infected host cells, the incubation period is short and the mutation rate can be rapid. Symptoms of ZIKV infection are thought to be minor in adults, including a transient rash and arthralgia, and are similar to symptoms associated with infection by other flaviviruses such as Dengue (DENV) or Chikungunya.[91] ZIKV is usually detected from blood or urine samples with RT-PCR, which identifies the presence of viral RNA, or MAC-ELISA, which detects antibodies to viral components. RT-PCR only works as long as patients are actively infected with the virus, typically 3–5 days after infection and MAC-ELISA can yield false positives due to cross-reactivity with other flaviviruses like DENV.[117,118] ELISA testing of neonatal sera and cerebrospinal fluid for ZIKV-specific IgM has confirmed congenital ZIKV infections in newborns with microcephaly.[119] Most recently, a reverse transcription loop mediated isothermal amplification method for detecting ZIKV has been reported as a more rapid and field-safe test for ZIKV infection.[120]

and were 40% smaller than mock-treated controls. Because none of these defects were observed when cells were exposed to DENV, the authors concluded that ZIKV, not flaviviruses in general, promote aberrant proliferation, differentiation, and death in neural stem and progenitor cells.[107] Coupled with analyses of human microcephaly patients, these two foundational studies indicate that neuronal progenitors are especially susceptible to ZIKV infection, with data supporting the hypothesis that loss of neural progenitors, especially during early development, underlies dramatic changes in brain size.

Despite the power of organoids and cell culture, animal models provide a vital tool for fully understanding ZIKV and developing methods that will combat infection. Studies have shown that laboratory mice respond to ZIKV infection differently depending on mouse strain and mode of infection. For instance, wild-type C57BL/6 mice can be infected by Zika (e.g., Ref 93), but the virus has only been detected in pups of mothers infected by vaginal swab.[125] A number of studies, however, provide evidence that C57BL/6 mice become more susceptible to ZIKV

infection upon suppression of interferon signaling. Specifically, disrupting the function of interferon α/β either through genetic deletion[87,125,126] or administration of an interferon-blocking antibody during early development[126] confers susceptibility to ZIKV. When female mice lacking the receptor for interferon α/β, IFNAR, were crossed to wild-type male mice, these $Ifnar^{-/-}$ pregnant mothers showed signs of placental damage and their $Ifnar^{+/-}$ pups exhibited extensive spread of Zika virions and brain defects consistent with microcephaly.[127] Moreover, direct vaginal infection with ZIKV in $Ifnar^{-/-}$ mothers can be vertically transmitted to developing pups that often die in utero due to extensive viral-induced morphogenetic defects.[125] Further supporting a protective role for interferon signaling, mice in which the receptors for both interferon α/β and γ are genetically deleted (AG129 mice), are even more susceptible to ZIKV infection.[128] When adult AG129 mice are injected with Asian strains of ZIKV, they die a week after infection from viral-induced pathologies (including extensive cell death) in the brain and muscle. These mice were also susceptible to ZIKV transmitted from eye secretions and homogenates.[64] To further investigate the role of interferon signaling in the context of ZIKV, adult mice lacking interferon regulatory factor were infected with ZIKV. In these mice, ZIKV infection negatively affected neurogenic regions, the anterior SVZ of the forebrain and the subgranular zone (SGZ) of the hippocampal dentate gyrus.[87] Cells in these regions showed elevated caspase activation and reduced proliferative potential. Creating a susceptible mouse model by disrupting key components of the immune system may obfuscate the exact mechanisms by which ZIKV infects the host. These models do, however, allow for recapitulation of the ZIKV-associated phenotypes, and provide data suggestive of a protective role for interferon signaling, at least in mice.

Other inbred mouse strains have been tested for susceptibility to ZIKV-linked neural developmental defects. ICR mice, which are often used as a model for carcinogen-induced colon cancer, are also providing insight into how ZIKV causes microcephaly.[51] Consistent with in vitro findings, ZIKV injection into the brain of ICR mice induced activation of the apoptotic caspase and immune response pathways, cell cycle arrest, and increased expression of candidate flavivirus entry receptors. Specifically, ZIKV infection of RG and oRG triggered apoptosis of all classes of cells examined, including cycling NPCs, immature, and mature neurons.[51] Another mouse strain, SJL, which has functional interferon signaling and is used to model spontaneous myopathies, may be most

useful for studying vertical transmission of ZIKV as pups born to ZIKV-infected mothers have Zika virions throughout their bodies and concentrated in their brains.[93] These pups exhibit brain and eye phenotypes similar to ZIKV-infected fetuses, including cortical malformations, fewer cells, and apoptotic remnants throughout the CNS.

In addition to murine models, nonhuman primates such as rhesus macaque monkeys are particularly important for studying primate-specific consequences of ZIKV infection and for vaccine development. Several recent studies revealed that monkeys could be infected with ZIKV, highlighting the multiorgan repercussions of ZIKV infection (e.g., Ref 129). Two other studies provide additional insight into ZIKV infection in primates and offer evidence for protective immunity from previous infection with different[130] or the same[131] ZIKV strains. Nonhuman primate models are also important for understanding how the primate immune system responds to infection with multiple flaviviruses, especially in the context of vaccine development (see final section for more details).

In all of the animal models used to date, various routes of ZIKV infection have been used throughout these models, including vaginal swab,[125] transmission via ocular secretions,[64] and injections into multiple sites including the footpad,[128] intraperitoneal space,[126] retroorbital space,[87] or cerebellar ventricles (e.g., Ref 51). While the injection into the cerebral ventricles ensures that virions reach neural tissues of interest, peripheral injections align more closely with the mosquito–human route of administration. Vaginal swab mimics sexual transmission. Existing mouse and primate models will likely further our understanding of ZIKV pathology and identify targets for the development of vaccines and prophylactic treatments.

OPEN QUESTIONS AND FUTURE DIRECTIONS

Current evidence demonstrates that ZIKV induces microcephaly in humans when passed vertically from mother to fetus, particularly when this transfer occurs during the first trimester of pregnancy. Although a flurry of recent experimentation has begun to identify potential mechanisms by which ZIKV-induced microcephaly emerges, much work remains. Additional research is needed to understand the molecular properties and infection mechanisms of ZIKV. In particular, the genes that are dysregulated by ZIKV infection should be more closely examined. While studies have reported a host of genes that are differentially expressed after Zika infection (e.g., Refs 52,93,104,121), it remains unclear how these catalogued changes effect ZIKV pathogenesis. In particular, it will be interesting to learn how virus-induced gene expression changes influence how ZIKV gains access to immune privileged areas, whether by crossing placental and BBBs or by unique transmission routes. Moreover, identifying the cell surface receptor(s) for ZIKV will provide valuable information for halting ZIKV infection, especially in particularly vulnerable tissues.

Another crucial goal of on-going work will be to develop treatments and prophylactic measures to halt the spread of ZIKV. Based on the protective capabilities of interferons (as discussed earlier), it will be important to examine whether boosting interferon signaling can reduce the susceptibility of embryos to Zika infection. In addition, several ZIKV vaccines have been tested in mice and rhesus monkeys and appear to provide protection from microcephaly-associated ZIKV strains,[132–134] and Phase I clinical trials are beginning to evaluate the safety and efficacy of a ZIKV vaccine.[135] Despite this progress, immunogenic crosstalk between ZIKV and DENV complicate the development of effective vaccines for each virus. The amino acid sequence of ZIKV E protein, which is associated with viral entry and host defense, is more similar to that of DENV than any other flavivirus.[136] Thus, monoclonal antibodies produced in response to DENV bind weakly to Zika virions at levels that cause antibody-dependent enhancement (ADE) rather than virus neutralization.[137] Epidemiological analyses will be necessary to determine whether infection with any of the four DENV serotypes raises the risk of ZIKV infection. One recent study suggested that exposure to another flavivirus, HSV-2, raises susceptibility to infection by ZIKV.[138] Given this risk, and the fact that immunity to one DENV serotype does not protect against other serotypes and may increase risk of infection, it is therefore paramount to develop a vaccine that is cross-protective, with one potential target being the fusion loop of the E protein.[136]

Scientists are also identifying pharmacological approaches to treat Zika infection. One large-scale screen identified 20 potential candidate FDA-approved drugs that may stymie ZIKV infection.[139] Another large-scale screen identified azithromycin, a macrolide antibiotic, as capable of reducing both viral proliferation and ZIKV-induced cytopathy in developing human brain tissues.[80] In addition, chloroquine, a drug commonly used to prevent malarial infection, can reduce ZIKV infection in several cell lines regardless of ZIKV strain.[88] Finally, the

mechanism by which ZIKV neutralizing antibodies work is also being actively investigated.[140,141] Additional studies are needed to understand how these various treatments work and whether they will be efficacious in the clinic.

From all this recent work, most of it within a short 16-month period, it is clear that basic research into the molecular mechanisms of Zika infection will pave the way for the development of effective treatments and protective measures.

FURTHER READINGS

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