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Research Paper

Infectivity of Immature Neurons to Zika Virus: A Link to Congenital Zika Syndrome

Brandon W. Hughes, Krishna C. Addanki, Ahila N. Sriskanda, Ewen McLean, Omar Bagasra *

Claflin University, South Carolina Center for Biotechnology, 400 Magnolia Street, 29115 Orangeburg, SC, United States

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ABSTRACT

Background: Epidemiological data strongly suggest that microcephaly cases in Brazil are associated with the ongoing epidemic of Zika virus (ZIKV). In order to further solidify the possible link, we investigated the infectivity of ZIKV using various neuroblastoma (NB) cell lines.
Methods: Six undifferentiated, two terminally differentiated and two retinoic acid (RA) –induced, partially differentiated cell lines were exposed to ZIKV strain PRVABC59, which is genetically similar to the French Polynesia strain, with 97–100% genetic homology to the current ZIKV strain found in Brazil. All infections were confirmed by real-time PCR (RT-qPCR), immunofluorescence assay (IFA) probing with anti-flavivirus E antibody, and evaluation of cytopathic effects.
Findings: ZIKV infected all six undifferentiated NB cell lines. In five out of six NB cell lines, between 90 and 70% cells were positive by IFA whereas for one cell line, CCL-127, ~80% of cells were positive for ZIKV as determined by IFA but showed persistent infection. Two differentiated cell lines, JFEN and T-268, were highly resistant to ZIKV with <1% of the cells being susceptible, as determined by IFA and confirmed by qRT-PCR. Two retinoic acid (RA)-induced NB partially differentiated cell lines showed no difference in permissiveness as compared to their undifferentiated mother cell lines.
Interpretation: These findings strengthen the reported association between high incidences of microcephaly and ZIKV infection in newborns in Brazil. Our results suggest that the undifferentiated neurons are highly permissive to ZIKV infection, as one would expect during the early stages of neurogenesis in fetal brains; whereas differentiated neurons, representative of adult brain neurons, are relatively resistant to the virus, which explains the rare occurrence of neurological complications in adults infected with ZIKV. Our studies confirm the neurotropism of the ZIKV strain closely related to the current epidemic in Latin America.

1. Introduction

Zika virus (ZIKV) is a positive-sense, single-stranded RNA member of the family *Flaviviridae* (Mlera et al., 2014). The natural transmission cycle of ZIKV predominantly involves mosquito vectors from the *Aedes* genus and monkeys (Hayes, 2009), although ZIKV antibodies have been discovered in a number of other animals (Henderson et al., 1968; Kaddumukasa et al., 2015), including rodents (Darwish et al., 1983). Humans generally act as infrequent hosts (Kaddumukasa et al., 2015), but may represent a primary amplification and reservoir species in endemic areas (Darwish et al., 1983). The first report of clinical manifestations of ZIKV was incidental to an outbreak of jaundice in Afikpo, Nigeria in 1954, which was suspected of being yellow fever (MacNamara, 1954). The virus causes an acute febrile illness, symptomatically similar to dengue, and is characterized by mild headache, fever, maculopapular rash, joint and back pain, and general malaise, sometimes accompanied by conjunctival hyperemia, anorexia, dizziness, diarrhea and constipation (Dick et al., 1952; Bearcroft, 1956; Simpson, 1964). The incubation period is between 3 and 12 days and symptoms may last for 2–7 days. Only ~20% of presenting patients infected with ZIKV exhibit symptoms and the virus has never been reported to cause hemorrhagic fever or death, often resulting in misdiagnosis as dengue (Dick et al., 1952; Bearcroft, 1956; Simpson, 1964).

In early 2015, a serious ZIKV outbreak was recorded in Camaçari, Bahia, Brazil (Plourde and Bloch, 2016; Cerbino-Neto et al., 2016) and this was later followed by similar illnesses in five neighboring states leading the Ministry of Health to issue a ZIKV alert in April (Rasmussen et al., 2016; Chan et al., 2016). By the end of the year the virus had spread through 19 states, many in the northeastern part of the country. Subsequently, the virus migrated throughout most other South and Central American countries (Chan et al., 2016) and the pandemic swept into the Caribbean with autochthonous transmission in

* Corresponding author.
E-mail address: obagasra@claflin.edu (O. Bagasra).

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at least six island states. The rapid movement of ZIKV, together with troubling reports of association between ZIKV and infants born with microcephaly, prompted the WHO to issue an "International Health Emergency" on February 1, 2016. Nevertheless, a direct relationship between ZIKV infection and increased incidence of neurologic disorders and microcephaly have emerged, and now there is irrefutable evidence to support a strong link between ZIKV infection and the observed neurologic defects in newborns (reviewed in 12–14).

Until recently, there have been few reports demonstrating a direct link between ZIKV infection and congenital Zika syndrome (CZS). Of three new studies, the recovery of a full length ZIKV genome from an infant with microcephaly, recovery of ZIKV or positive RNA from amniotic fluid of an abnormal fetus, and recovery from brain tissues of two fetuses from ZIKA infected mothers provide convincing evidence for such a link (reviewed in 12–13). Moreover, in certain instances, impacted regions of the Brazilian outbreak registered a >10-fold increase in occurrence of microcephaly; a magnitude surge that cannot be explained by random clustering. Earlier ZIKV outbreaks in French Polynesia too have been retrospectively strongly associated with increased occurrence of microcephaly (European Centre for Disease Prevention and Control (ECDC), 2015).

A major cause for concern is that ZIKV and related viruses may adapt to transmission through species of mosquito other than *Aedes aegypti*. For example, *Aedes albopictus* is well established around the Mediterranean in Europe and is present in at least 32 states in the US (Cauchemez et al., 2016). This species could provide a vector for the onward transmission of ZIKV across both continents that receive viremic returning travelers (Zammarchi et al., 2015), since there is laboratory evidence that *A. albopictus* is a competent vector for the virus (Wong et al., 2013). Moreover, changes in climate may contribute to range expansion for ZIKV and its vectors (Kraemer et al., 2015), and autochthonous cases in temperate regions may occur even in absence of a competent vector through sexual transmission (Venturi et al., 2016).

At present, no specific vaccines or treatments for ZIKV are available and, although the possibility exists that present flavivirus vaccine technologies could be adapted to ZIKV (Rochlin et al., 2013), it is important to investigate the molecular pathogenesis of the virus. Here, we show that six human NB cell lines, which behave similarly to a developing human fetal brain, can be infected with ZIKV. We also show that human differentiated NB neuronal cell lines (JFEN and T-268) are significantly resistant to ZIKV. This report illustrates the differential permissiveness of immature versus mature human neuron infections from a strain that is genetically similar to the ZIKV strain responsible for the epidemic in Brazil.

2. Material and Methods

2.1. Reagents and Cell Lines

Five NB cell lines were purchased from ATCC (New York, NY). CRL-2267, CCL-127, CRL-2271 (of male origin) CRL-2266, and CRL- 2149 (of female origin) were cultured in Eagle's Minimum Essential Media (EMEM) (ATCC, Manassas, VA) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), and L-Glutamine-penicillin-streptomycin solution (designated complete media) (Sigma, St. Louis, MO) at 37 °C, 5% CO_2. SMS-KCNR (of male origin) was purchased from Children's Oncology Group (Texas Tech University) and was cultured in RPMI-1640 (Sigma). Two terminally differentiated olfactory neuroblastoma cell lines, T-268 and JFEN, were a kind gift from Timothy J. Triche (Department of Pathology, Children's Hospital of Los Angeles, Los Angeles, CA 90027; 21). The stock cell cultures were grown in 25 mL or 75 mL flasks (Thermo-Scientific, Nunc, Rochester, NY). ZIKV strains PRVABC59 and MR 766 were provided by the CDC (kind gifts from Brandy Russel, Fort Collins, CO) and flavivirus envelop antibody (FE1), which cross-reacts with ZIKV, was purchased from Invitrogen (Cat. # MA1-71258). Two NB cell lines CRL-2266 and CRL-2267 were exposed to retinoic acid (RA) at 1 μM final concentration for 48 h (Breitman et al., 1980). Both cell lines exhibited partial differentiation as measured by morphologically and by immunostaining with anti-tubulin antibody.

2.2. Propagation of ZIKV

ZIKV strain (KU501215) was propagated in Vero cells (ATCC). The cells were grown in EMEM to 70% confluency in 25 mL tissue culture flasks (Nunc Inc., USA). Following removal of the culture media, virus inoculum was added to give a multiplicity of infection (MOI) of 0.1 to 0.05/cell or 1.0 to 1.5/cell. Flasks were incubated at 37 °C, 5% CO_2 with gentle agitation for 30 min. After incubation, 5 mL of complete media was added and the cells were maintained for 5–9 days or until cells exhibited cytopathic effects (CPE). Cells were trypsinized and cryopreserved in 50% FBS and 10% DMSO at −80 °C and used in subsequent studies.

2.3. Infection of Neuroblastoma Cell Lines With ZIKV

To examine the permissiveness of NB cell lines to ZIKV, all 6 undifferentiated cell lines and two olfactory NB cell lines, T-268 and JFEN (Sorensen et al., 1996), were grown in 25 mL tissue culture flasks to ~70% confluency, media was removed and immediately exposed to an estimated 0.1 to 0.05 MOI or 1.0 to 1.5 MOI in 1.0 mL serum free media. For maximum infectivity, the flasks were intermittently gently agitated for 1 h and then 5 mL of pre-warmed media was added. Control cultures were treated identically, except no ZIKV was added to the inocula. The cells were incubated at 37 °C, 5% CO_2. The cell cultures were observed every 12 h for CPE and images recorded on a digital microscope camera. Each experiment was repeated at least three times for low MOI and twice for high MOI infected cells.

2.4. Immunofluorescence Assay (IFA)

For immunocytochemical studies, each cell line was grown in 8-well chamber slides (Thermo Scientific, Nunc, Rochester, NY) with ~1×10^5 cells in 0.3 μL of media for 4–7 days. For seeding the NB cell lines, the stock cell cultures grown in the flasks were gently washed once with 1× PBS followed by trypsinization until single cell suspension and inactivation of trypsin with 1 mL FBS. The cells were counted on a hemocytometer chamber and adjusted to 1×10^5 cells/mL. The 8-well chamber slides were labeled, 100 μL of cells were then added to each well of the respective 8-well chamber slides, and 1 μL of fresh media was added containing 0.1–0.05 MOI of the virus. After 1 h of incubation, the cells were washed gently and 500 μL of fresh media was added. After 48 h culture, the media was removed from the slides and the cells were fixed by adding 800 μL of Streck Tissue Fixative (STF, Streck Laboratories Inc., LA Vista, NE), a non-ionic fixative, in each well and allowed to set overnight at 4 °C. The wells were then washed gently three times using 1× sterile Phosphate Buffered Saline (PBS) (Fisher Scientific, Fair Lawn, NJ) and then soaked with the blocking agent (containing 2% Bovine Serum Albumin (BSA) in 1× PBS) for 10 min. ZIKV was detected by utilizing mouse monoclonal antibody to flavivirus (Cat. # MA1-71258, Invitrogen). All antibody dilutions were carried out in 2% BSA in 1× PBS. The primary Ab was diluted to 1:10 in blocking buffer. In each chamber, 100 μL of diluted primary Ab was added to each well. The slides were incubated at 4 °C overnight in a humidified chamber and then washed three times in 1× PBS. Then, goat anti-mouse fluorescein conjugated secondary Ab was used at a working dilution of 1:40 with 100 μL in each well. The slides were incubated for 1 h at 37 °C in humidified chambers and then washed three times with PBS with 10 min incubation each time. The tops of the 8-well chamber were carefully removed without disturbing the fixed cells. The cells were washed in PBS three times and then mounted with glass coverslips, using a drop of mounting solution containing 50% glycerol, 50% PBS. The slides were observed at resolutions 10×, 40×, and 1000× (with oil) using an Olympus BX51 fluorescent microscope. Each experiment was repeated at least three times with low MOI and two times with high MOI of ZIKV.

The slides were analyzed independently in a blinded fashion by two different observers.

2.5. Real-Time PCR (RT-PCR)

The ZIKV RNA copies were quantified using one-step RT-PCR. Viral RNA was extracted from culture pellet using GenElute Mammalian Total RNA Miniprep kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. Two sets of primer pairs were designed to target the 5′UTR region of the virus genome (Forward primer, ZIKV_F, 5′-TTGGTCATGATACTGCTGATTGC-3′ and reverse primer, ZIKV_R, 5′-CCTTCCACAAAGTCCCTATTGC-3′) and (Forward primer, Zika E_F, 5′ AAGTTTGCATGCTCCAAGAAAAT-3′ and reverse primer, Zika E_R,-5′ CAGCATTATCCGGTACTCCAGAT-3′). The reactions of qRT-PCR were carried out using iScript One-step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA) and quantification was performed using ABI 7500 real-time cycler (Applied Biosystems, Foster City, CA). The thermal cycling profile of this assay consisted of a 10 min cDNA synthesis step at 50 °C, 5 min of iScript reverse transcriptase inactivation at 95 °C, followed by 35 cycles of PCR at 95 °C for 10 s and a step of a single fluorescence emission data collection at 59 °C for 30 s. Each experiment was repeated three times with low MOI and two times with high MOI of ZIKV. Results were analyzed using HID Real-Time PCR Analysis Software v1.2 (Thermo Scientific, Waltham, MA). Relative degree of ZIKV replications in different cell lines were calculated using cycle threshold values (C_t).

3. Results

3.1. Real-Time PCR and Immunofluorescence Assay

In order to determine incidence of ZIKV infection in NB cell lines, a presence/absence assay was conducted by RT-PCR. Analysis for presence of ZIKV showed positive infection of all six undifferentiated NB cell lines. The same, uninfected, cell lines were used as controls, which showed an absence of ZIKV RNA (Table 1). Cell lines CRL-2266, CRL-2267, CRL-2271, CRL-2149, CCL-127, and SMS-KCNR showed positive infection by ZIKV, whereas the controls were negative for ZIKV. There were no significant differences in the degree of permissiveness of ZIKV between NB cell lines of male and female origin (Table 1). Statistical analyses were carried out using GraphPad Prism software (San Diego, CA) by unpaired Student's *t*-test. An immunofluorescence assay, with anti-flavivirus envelope IgG, confirmed the RT-PCR results (Table 1 and Fig. 1). Two differentiated neuronal cell lines, JFEN and T-268, showed significant resistance to ZIKV, as evaluated by RT-PCRs and IFA (Fig. 1). Also, when cell lines CRL-2266 and CRL-2267 were partially differentiated via 48 h RA exposure, there were no significant differences in amplification, as compared to the undifferentiated NB cell lines infected with ZIKV. RA induced partial differentiation in the progenitor NB cells where ~31% of the neurons exhibited elongated axons in CRL-2266 and ~22% of the neurons showed elongated axons in CRL-2267 (Supplemental Fig. 1). The RT-PCR results also confirmed that there was no significant decrease in permissiveness to ZIKV as compared to the immature parental NB cell lines. Suppressed growth rate and morphological evidence, such as increased neuritic processes, confirmed partial differentiation. The presence or absence of ZIKV in all of the aforementioned cell lines was further confirmed by utilization of two sets of primer pairs by RT-PCR and agarose gel electrophoresis, in which NB cells showed presence of ZIKV RNA produced by RT-PCR with expected bands of 76 and 72 base pairs. There were no significant differences in the percentage of cells infected with low MOI (0.1–0.05 MOI) versus high MOI (1.0 to 1.5), suggesting that permissiveness is related to the receptor-mediated entry of ZIKV (Hamel et al., 2015).

Table 1
Percent of ZIKV-infected neuroblastoma cells as determined by immunofluorescence assay and confirmed by RT-PCR.

Cell line	ZIKV+	RT-PCR	Remarks
CRL-2266	81%–85%	4+[a]	Immature, progenitor neurons
CRL-2267	67%–72%	4+	Immature, progenitor neurons
CRL-2271	84%–93%	4+	Immature, progenitor neurons
CRL-2142	90%–97%	4+	Immature, progenitor neurons
CCL-127	83%–86%	4+	Immature, progenitor neurons
SMS-KCNR	81%–89%	4+	Immature, progenitor neurons
JFEN	<1%–3%	1+	Differentiated neurons
T-268	<1%	1+[b]	Differentiated neurons
CRL-2266-RA	53%–78%	4+	Partially differentiated with ~31% of cells with long axons
CRL-2267-RA	61%–69%	4+	Partially differentiated with ~22% of cells with long axons

[a] 4+ is designated when the amplification line for a specimen crosses the threshold between 15 and 20 cycles.
[b] 1+ is designated when the amplification line for a specimen crosses the threshold between 33 and 35 cycles.

3.2. Morphological Study

All the NB infected cell lines were observed twice a day post-infection. The CPE was discernable within 24 h post-infection, when the neurons began to round up and float on top of the monolayers. We were surprised by the speed with which ZIKV infection spread throughout the monolayer, within 48 h post infection, and a significant percentage of cells in almost all the cell lines showed cell death, as measured by the Eosin-Y viability test (Supplemental Fig. 2). The infection with ZIKV produced acute lytic effects in all six immature NB cell lines. These cell lines exhibited CPE and neurons with highly abnormal morphology, including neurons showing elongation of cells, vacuolation within the cytoplasm, enlargement of the neuronal cell body, shortening, thinning or abnormal increase of axonal length, change in cellular diameter, central chromatolysis, and other abnormal cellular morphology (Fig. 2). The surviving immature cells were re-cultured in fresh media, and only one cell line, CCL-127, began to replicate but exhibited obvious evidence of CPE, including occasional giant cells (Fig. 2). This cell line has been maintained for the last 3 months. It shows persistent infection that is documented by RT-PCR and IFA (data not shown). All six NB cell lines showed low viability after 48 h, whereas two mature cell lines, JFEN and T-268, expressed no clear morphological changes and appear to be resistant to the cytopathic effects of ZIKV. Therefore, even 12 weeks post-infection, the cells grew at a similar growth rate to uninfected cells. The two mature NB cell lines appeared normal and without any apparent CPE. Immunostaining with flavivirus E Ab, as well as RT-PCR, confirmed the relative resistance to ZIKV (Table 1; Fig. 1).

In summary, in all types of NB cell lines, ZIKV infection induced marked and significant changes of the neurons and caused neuronal proliferation, central chromatolysis, enlargement of the neuronal cell body, shortening or abnormal increase and thinning of axonal length, syncytia formation, and neurostimulations or selective neurocytotoxicity.

4. Discussion

Since January 2007 ZIKV outbreaks have been documented in 61 countries and, in 57 of these, the epidemic continues (http://www.who.int/emergencies/zika-virus/situation-report/21-april-2016/en/). The speed at which the virus has spread serves to highlight inherent deficiencies in emergency preparedness and response protocols for many infected countries; especially as this relates to emerging diseases. Mounting evidence points to ZIKV being sexually transmitted and it has been associated with a variety of neurological syndromes including Guillain-Barré Syndrome (GBS), meningitis, myelitis, and meningoencephalitis (European Centre for Disease Prevention and Control (ECDC), 2015; Cauchemez et al., 2016). The existence of a link between fetal defects, microcephaly, and ZIKV infection during pregnancy is strong (Rasmussen et al., 2016). The results presented herein serve to

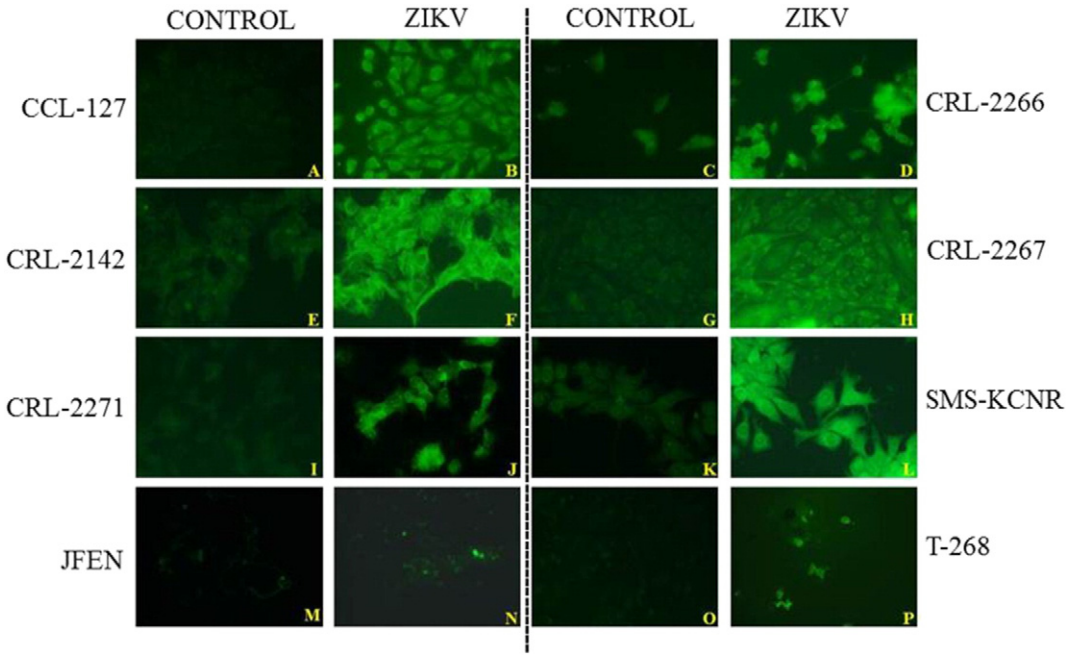

Fig. 1. Immunofluorescence assay utilizing a cross acting flavivirus monoclonal antibody to detect ZIKV envelope antigen expression on the cell surface of ZIKV infected and uninfected neuroblastoma cell lines. (Top row, left to right) A) CCL-127 uninfected; B) CCL-127 ZIKV infected; C) CRL-2266 uninfected; D) CRL-2266 ZIKV infected; E) CRL-2142 uninfected; F) CRL-2142 ZIKV infected; G) CRL-2267 uninfected; H) CRL-2267 ZIKV infected; I) CRL-2271 uninfected; J) CRL-2271 ZIKV infected; K) SMS-KCNR uninfected; L) SMS-KCNR ZIKV infected; M) JFEN uninfected; N) JFEN ZIKV infected; O) T-268 uninfected; P) T-268 ZIKV infected.

confirm the latter possibility since, in all types of NB cell line, ZIKV infection induced marked and significant CPE in neurons.

We did not find any difference in the percent of NBC infected with low or high MOI by IFA or RT-PCR. ZIKV could potentially use DC-SIGN and phosphatidylserine receptors, such as T-cell immunoglobulin and mucin domain (TIM) and TYRO3, AXL, and MER (TAM; Hamel et al., 2015; Meertens et al., 2012), or perhaps CD300a (Simhadri et al., 2012), to gain access or adhere to cells. Accordingly, our results suggest that available receptors, if this represents the sole mechanism of entry, were fully occupied even at the low MOI. As illustrated herein, ZIKV is

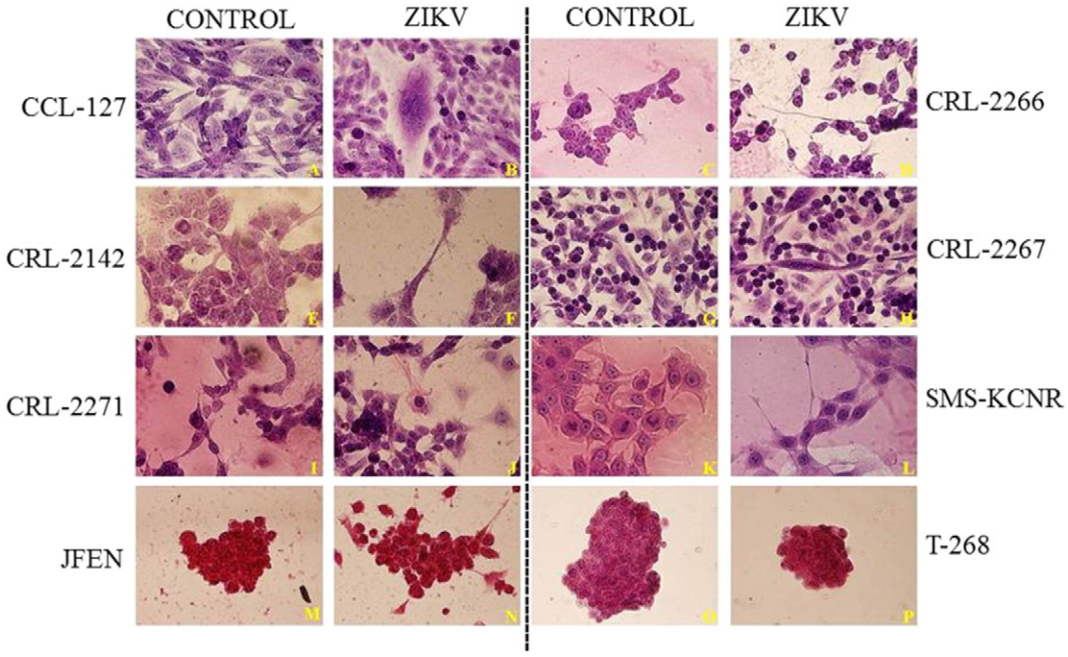

Fig. 2. Morphological analyses by utilizing H&E staining. (Top row, left to right) A) CCL-127 uninfected; B) CCL-127 ZIKV infected; C) CRL-2266 uninfected; D) CRL-2266 ZIKV infected; E) CRL-2142 uninfected; F) CRL-2142 ZIKV infected; G) CRL-2267 uninfected; H) CRL-2267 ZIKV infected; I) CRL-2271 uninfected; J) CRL-2271 ZIKV infected; K) SMS-KCNR uninfected; L) SMS-KCNR ZIKV infected; M) JFEN uninfected; N) JFEN ZIKV infected; O) T-268 uninfected, P) T-268 ZIKV infected. All the infected cells, except JFEN and T-268, are showing significant ZIKV infection induced changes of the neurons and showed neuronal proliferation, central chromatolysis, enlargement of the neuronal cell body, vacuolation within the cytoplasm, shortening or abnormal increase and thinning of axonal length, syncytia formation, and neurostimulations or selective neurocytotoxicity.

highly neurotropic, especially for immature, undifferentiated neurons; hence, once a pregnant woman becomes viremic, there is high potential that the infection will reach fetal brain neurons. It appears that the most vulnerable stage of infection for the fetus is before 17 weeks of gestation, after which the risk to the unborn child is low (Carneiro and Travassos, 2016). TYRO3, a member of the TAM family of receptor tyrosine kinases (RTKs), is abundant on the surface of neurons and appears to be the prime target for ZIKV (Rothlin and Lemke, 2010), a possibility that seems justified given that the brains of suckling mice infected with ZIKV express neuronal degeneration, cellular infiltration, and softening, with evidence of virus replication in astroglial cells and neurons on histopathological examination (Rothlin and Lemke, 2010; Bell et al., 1971). However, we speculate that the entry receptors for ZIKV are absent or significantly downregulated in the mature, terminally differentiated neuroblastoma cell lines, blocking the viral entry to these neurons. This possibility would need further investigation (Bell et al., 1971).

Dysregulation of neuronal cell cycling following in vitro ZIKV infection has also been observed using flow cytometric and transcriptome analyses by Tang et al. (2016). Interestingly, the speed of cell contagion in the study described herein was more rapid than that observed by Tang et al. (36 h versus 66–72 h). This may have resulted due to differences in experimental design, cultured cells employed (induced pluripotent stem cells derived from human skin cells versus human fetal brain progenitor neurons derived from childhood neuronal crest cells (neuroblastoma; 30). Also we used the ZIKV strain that is genetically similar to the current Brazilian strain of ZIKV (MR766 versus PRVABC59; Zhu et al., 2016; Nunes et al., 2016). Other morphological changes associated with ZIKV infection included syncytia and giant cell formation, which generally results due to the expression of viral fusion proteins (Lee and Bowden, 2000), and central chromatolysis, wherein chromatin and cell nuclei were dislocated to the cell's periphery, indicating general injury. Enlargement of the neuronal cell body and shortening or abnormal increase and thinning of axonal length were also observed. The latter abnormalities represent common defects encountered during other viral infections including rubella, cytomegalovirus, herpes, and West Nile virus (Hamel et al., 2015; Stewart et al., 2013; O'Leary et al., 2006; Watrin et al., 2016).

There was no difference between male and female NB cell lines, even though there was male predominance with a sex ratio of 2.82 in the incidence of Guillain-Barré Syndrome (GBS) during the ZIKV epidemic in French Polynesian Islands (Whitman and Greer, 2009). Furthermore, we did not observe a significant difference between the undifferentiated and partially differentiated NBCs (RA-induced neurons). This may suggest that, after RA exposure, the cell lines were not terminally differentiated and still need additional steps for terminal differentiation (Kovalevich and Langford, 2013).

The findings described here may explain the permissiveness of developing immature progenitor neurons, as well as the relative resistance of mature neurons found in adult human brains, to ZIKV. Our studies also point to a relative vulnerability of replicating and regenerating neurons to ZIKV, such as the olfactory and dentate gyrus of the adult human brain (Whitman and Greer, 2009). Of note, the French Polynesian outbreak of ZIKV was remarkable because 74 infected individuals were presented with neurological symptoms, of which 47 were later diagnosed with GBS (Cauchemez et al., 2016; Hamel et al., 2015). Even though GBS is generally considered to be the result of autoantibodies to the peripheral nervous system antigen, inflammation of dentate gyrus has been reported (Müller et al., 2003). This may explain why GBS is rather infrequent, whereas microcephaly is more common during ZIKV infections. Of note, we have to also realize that warming climate may increase the range of mosquito and other vector-mediated transmission of many diseases, including ZIKV (Liu-Helmersson et al., 2016).

The limitation of the current study is that we have utilized NBC lines and not actual human prenatal subjects, which would be unethical. Also, there are infinite differences between in vivo development of progenitor cells in a fetal brain and in the in vitro environment, as employed herein. However, we believe that despite these limitations, we present a clear picture of permissiveness of undifferentiated versus terminally differentiated neurons.

Author's Contributions

BWH and KCA contributed equally in preparation of the manuscript. OB conceived the idea, planned the experiment, and performed some of the experiments. BWH, KCA, and ANS also performed some of the experiments. EM wrote some of the manuscript. All authors assisted in preparation of the manuscript.

All authors declare no conflict of interest.

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Appendix A. Supplementary Data

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