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West Nile virus infection of the placenta

Justin G. Julander [a], Quinton A. Winger [b], Lee F. Rickords [b], Pei-Yong Shi [c], Mark Tilgner [c], Iwona Binduga-Gajewska [c], Robert W. Sidwell [a], John D. Morrey [a,*]

[a] *The Institute for Antiviral Research, Utah State University, Logan, UT 84322, USA*

[b] *Department of Animal, Dairy, and Veterinary Sciences, Utah State University, Logan, UT 84322, USA*

[c] *Wadsworth Center, New York State Department of Health, Albany, NY 12208, USA*

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Abstract

Intrauterine infection of fetuses with West Nile virus (WNV) has been implicated in cases of women infected during pregnancy. Infection of timed-pregnant mice on 5.5, 7.5, and 9.5 days post-coitus (dpc) resulted in fetal infection. Infection of dams on 11.5 and 14.5 dpc resulted in little and no fetal infection, respectively. Pre-implantation embryos in culture were also infected with WNV after the blastocyst stage and the formation of trophectoderm. Green fluorescent protein (GFP) expression was observed in a trophoblast stem (TS) cell line after infection with a GFP-expressing WNV construct. However, no fluorescence was observed in differentiated trophoblast giant cell (TGC) cultures. GFP fluorescence was present in TGC cultures if infected TS cells were induced to differentiate. These results suggest that embryos are susceptible to WNV infection after the formation of the trophectoderm around 3.5 dpc through the formation of the functional placenta around 10.5 dpc.
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Keywords: West Nile virus; Placenta; Trophoblast; Intrauterine; Vertical transmission; Pregnancy

Introduction

West Nile virus (WNV) causes disease in man, including encephalitis, paralysis, and death (Anderson et al., 2004). WNV may also infect horses, dogs, cats, and alpacas, as well as other species such as alligators (Abutarbush et al., 2004; Austgen et al., 2004; Kutzler et al., 2004; Miller et al., 2003; Yaeger et al., 2004). The primary vector for human transmission is the mosquito, however, other modes of infection have been observed (Sardelis et al., 2001; Turell et al., 2001). Virus has been transferred in human patients by blood and organ transplantation, as well as by accidental laboratory infection (Laboratory-acquired West Nile Virus, 2002; Macedo de Oliveira et al., 2004; Wadei et al., 2004). Some animal species have become infected after ingestion of infected materials or contact, such as feather picking or grooming, with infected individuals (Banet-Noach et al., 2003; Miller et al., 2003; Odelola and Oduye, 1977).

Intrauterine infection of fetuses with WNV has been implicated (Intrauterine West Nile virus, 2002), but other reports of maternal infection with WNV during pregnancy have shown no evidence for morbidity of the fetus (Bruno et al., 2004). Many other WNV cases of maternal infection during pregnancy are under investigation (Interim Guidelines, 2004). A woman infected with WNV during pregnancy gave birth to a WNV-seropositive baby with chorioretinal scarring and some brain abnormalities that may have been due to maternal infection with WNV during the second trimester of gestation (Alpert et al., 2003). Fetal viral infections are generally transmitted from maternal viremia across the placenta to fetal circulation, so an understanding of viral interactions with the placenta is important (Kaplan, 1993).

Infection of mouse fetuses was recently demonstrated in our laboratory (Julander et al., 2005). In that study, dams infected with WNV 7.5 days post-coitus (dpc) had a high rate of passage of maternal virus to fetuses as compared to low frequency of fetal infection when dams were infected 11.5 dpc.

* Corresponding author. Mailing address: Biotechnology Center 305, Utah State University, 4700 Old Main Hill, Logan, UT 84322-4700, USA. Fax: +1 435 797-2766.

E-mail address: jmorrey@cc.usu.edu (J.D. Morrey).

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The placenta had elevated viral titer compared to other maternal organs regardless of the gestational time point of infection. Dams had high mortality and generally died prior to, or during, delivery unless treated with WNV-specific immunoglobulin. Immunoglobulin treatment allowed dams to conceive and raise pups.

Placental development is a dynamic process involving the interaction between invasive fetal-derived trophoblast cells and maternal decidual cells of the uterus (Fazleabas et al., 2004). At the blastocyst stage (3.5 dpc), just prior to implantation, surface cells of the embryo will differentiate into trophectodermal cells that will eventually give rise to the placenta and other extraembryonic structures (Cross et al., 1994). Trophoblast cells invade the maternal decidua during development and establish an interface between maternal and fetal blood for the transfer of nutrients to the developing fetus. The placental barrier between maternal and fetal blood is established in mice around 10.5 dpc and consists of one layer of mononuclear trophoblast cells (cytotrophoblast) and two layers of differentiated syncytial trophoblast (syncytiotrophoblast) (Georgiades et al., 2002). The placental barrier functions to allow selective transfer of nutrients and to inhibit the transfer of harmful materials, but this barrier may be breached by different chemicals or microorganisms (Koi et al., 2001a, 2001b).

A trophoblast stem (TS) cell line has been established by culturing blastocysts or early post-implantation trophoblasts in media containing fetal growth factor-4 (FGF-4), haprin, and fibroblast conditioned media (Tanaka et al., 1998). Upon removal of these components, the TS cells differentiate into other trophoblast cell types including trophoblast giant cells. The TS cell line serves as a model for the replicative and differentiated trophoblast cells of the placenta.

An understanding of the mechanism of WNV intrauterine infection may be important for preventing clinical cases as well as for the development of therapies to reduce fetal disease and associated symptoms. The objectives of this study were to delineate the timing of viral passage from infected dam to fetus and to identify the placental cell types susceptible to viral infection in vitro.

Results

Timing of fetal infection

To determine the gestational timing of fetal infection with WNV, timed-pregnant dams were challenged with WNV on 5.5, 7.5, 9.5, 11.5, and 14.5 dpc. Whole fetus, placenta, and maternal brain, kidney, and spleen were titered for WNV by infectious cell culture assay (Table 1). Virus was present in fetuses 6 days post-maternal challenge when dams were challenged on 5.5, 7.5, and 9.5 dpc. Fetuses from dams challenged 9.5 dpc had higher WNV titers than fetuses from dams challenged 7.5 dpc (Table 1). Little or no virus was present in fetuses from dams challenged 11.5 or 14.5 dpc. High WNV titers were present in the placenta regardless of gestational state at the time of infection. Maternal tissues had some detectable virus, but titers in maternal organs were much lower than titers in fetuses and placentas.

Infection of pre-implantation embryos

Groups of embryos were infected 1.5 dpc or 3.5 dpc with WNV-GFP and fixed 2 or 4 days post-infection (dpi). M16 media, used for culturing embryos, supported development of the embryos to blastocyst stage, but not further. Around 10% of the embryos died in culture (data not shown), and the remaining 90% were observed for fluorescence. A representative embryo from each time point is shown (Fig. 1). When embryos were infected 1.5 dpc, fluorescence was detected in embryos 5.5 dpc (Fig. 1B), but not 3.5 dpc (A) or in sham-infected controls (C). If embryos were cultured for 2 days and then infected on 3.5 dpc, fluorescence was observed in embryos 2 days post-infection on 5.5 (D), respectively, but not in sham-infected embryos (E). The fluorescence appeared in the trophectoderm of the blastocyst stage embryo (B, D). The trophectoderm, including some stem cells, differentiates around 3.5 dpc, therefore, infection with WNV-GFP coincided with the formation of the trophectoderm.

Table 1
West Nile virus (WNV) titers recovered from tissues and fetuses from mice infected at various times during gestation

Infected[d]	Necropsy[f]	Mean[a] virus titer ± SD[b] in maternal tissue samples (pos/total[c])				Mean virus titer ± SD in fetal samples (pos/total)	
(dpc[e])	(dpc)	Brain	Kidney	Spleen	Uterus	Fetus	Placenta
5.5	11.5	7.0 ± 1.8 (3/6)	5.5 ± 0.9 (2/6)	5.6 ± 0.3 (6/6)	6.5 ± 0.8 (6/6)	5.0 ± 0.8 (21/24)	8.4 ± 1.0 (24/24)
7.5	13.5	7.7 ± 2.0 (2/4)	<3.6 ± 0 (0/4)	6.2 ± 1.4 (3/4)	N/T[g]	7.0 ± 2.1 (23/31)	7.8 ± 1.1 (31/31)
9.5	15.5	6.7 ± 2.8 (2/5)	5.5 ± 0.4 (2/5)	6.6 ± 0.3 (5/5)	6.9 ± 0.4 (5/5)	7.6 ± 2.3 (15/15)	9.5 ± 0.5 (15/15)
11.5	16.5	6.8 ± 2.8 (2/7)	5.6 ± 0.5 (3/5)	N/T	N/T	5.8 ± 1.2 (3/28)	6.9 ± 1.2 (28/28)
14.5	19.5	<3.6 ± 0 (0/6)	5.7 ± 0.1 (3/6)	N/T	N/T	<3.6 ± 0 (0/30)	6.9 ± 0.8 (30/30)

[a] Mean virus titer is the average $TCID_{50}$/g tissue titer from positive samples that had viral titers above the levels of detection.
[b] Standard deviation.
[c] Tissue samples with detectable WNV titers per total samples tested.
[d] Day of gestation on which dam was challenged with WNV.
[e] Days post-coitus.
[f] Day on which tissue samples were harvested from infected dams.
[g] Not tested.

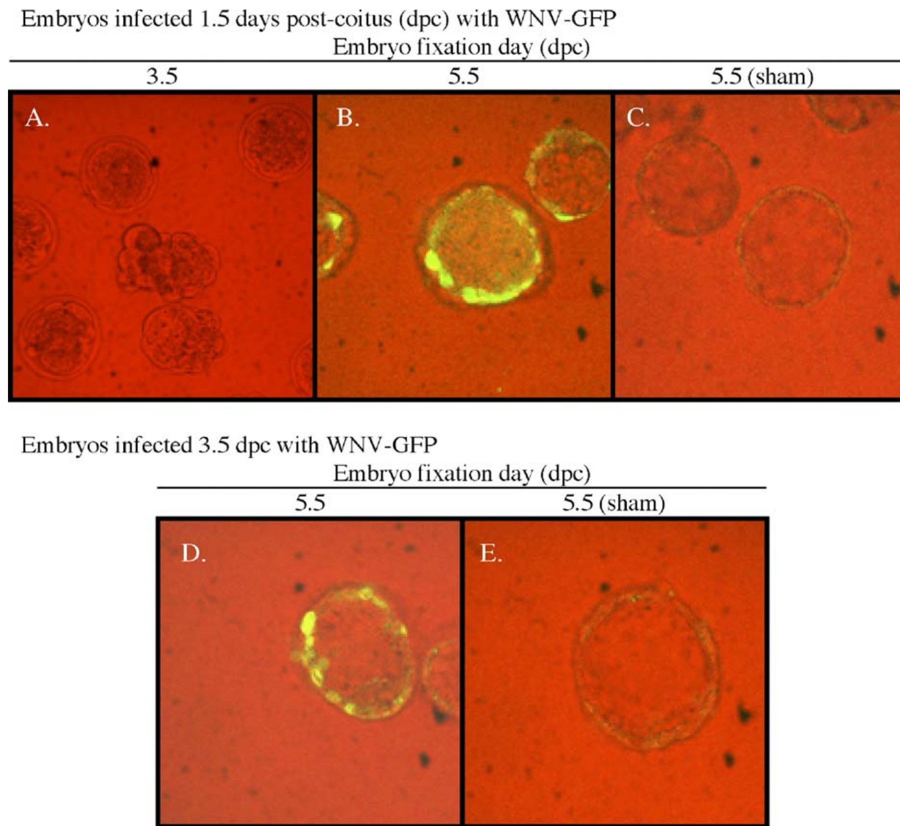


Fig. 1. Confocal microscopic images of embryos infected with a West Nile virus construct that expresses green fluorescent protein (WNV-GFP) on 1.5 days post-coitus (dpc) (A, B) or 3.5 dpc (D). Sham-infected controls were included (C, E). Embryos were harvested from timed-pregnant dams on 1.5 dpc. Embryos were fixed with 4% paraformaldehyde on 2 (A, D, E) or 4 days post-infection (dpi) (B, C). Panel size is 365 μm × 365 μm.

Infection of a trophoblast stem cell line

To determine the placental cell types involved in transplacental infection of the fetus, a mouse trophoblast cell line was used (Tanaka et al., 1998). In this cell culture model, TS cells are maintained in a replicative state by the addition of FGF-4, heparin, and FCM, and giant cell differentiation occurs after the removal of these components from TS cell cultures. TS cells were infected with WNV-GFP. TGC cultures were allowed to differentiate for 6 days prior to viral challenge. Transmission images (Figs. 2A, C, E, G, I, and K) were included above their respective fluorescent images to show the presence of TGC in the appropriate panels (white arrows). Fluorescence was observed in TS cells on 2 (data not shown), 4, and 6 dpi (B and D). Fluorescent intensity of infected TS cells increased from weak fluorescence in few cells on 2 dpi to strong fluorescence in many cells on 6 dpi in a time-dependent fashion. Vero cells infected in parallel also had increasing levels of fluorescence after 2 dpi (data not shown). Vero cells began showing cytopathic effect (CPE) 5 dpi, and few cells remained at 6 dpi, many of which had fluorescent emission. CPE was not as marked in TS cells as compared with Vero cells (A, C, and E). Most remarkably, no fluorescence was observed in differentiated TGC (G, I, and K) on 2, 4, or 6 dpi (H and J). However, if TS cells were infected and allowed to differentiate into TGC (Figs. 3A and C), fluorescence was observed in TGC cultures (B and D), which suggested that TGC were not restrictive for WNV replication, but they may be resistant to infection. Fluorescence was also less intense in differentiated infected TS cells as compared to fluorescence in infected TS cells maintained in a replicative state (G, H, I, and J). Induction of differentiation of TS cells 2 dpi resulted in significant CPE as compared to infected TS cells that were maintained in their replicative state (data not shown).

Characterization of GFP-expressing WNV

GFP-reporting WNV (Fig. 4A) was used to monitor viral infectivity during different stages of embryo development. It was previously shown that WNV containing a luciferase reporter is unstable. Multiple rounds of infections with such luciferase-expressing virus resulted in deletion of the reporter gene (Deas et al., 2005). Therefore, it is important to characterize the stability and growth kinetics of the GFP-WNV. We initially estimated the percentage of GFP-WNV in the virus stock harvested at day 4 post-transfection of BHK cells with RNA transcript (derived from the cDNA clone). Approximately 58% of the infectious viruses contained the GFP reporter, while 42% of the viral population was wild-type virus (data not shown). To prepare a homogeneous viral stock, we plaque purified the GFP-WNV in Vero cells for five rounds. The resulting viral stock was 100% GFP-positive (passage 0, Fig. 4B). However, when the homogeneous GFP-WNV was continuously passaged in Vero cells, wild-type virus without

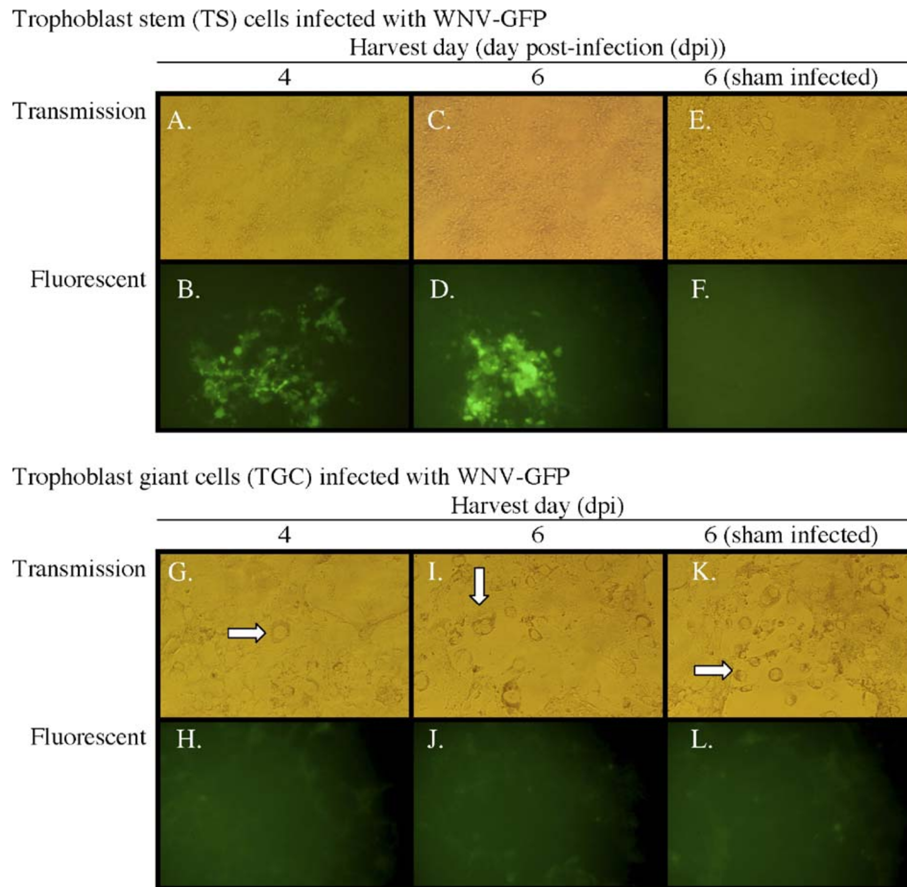


Fig. 2. Infection of trophoblast stem (TS) cells (A–F) and trophoblast giant cells (TGC) (G–L) with a West Nile virus construct that expresses green fluorescent protein (WNV-GFP). Fluorescence was observed in TS cells starting 2 days post-infection (dpi) and increasing in intensity 4 (B) and 6 (D) dpi. No fluorescence was observed in sham-infected TS cells (F). No fluorescence was detected in WNV-GFP-infected TGC (H and J) or in sham-infected TGC (L). Giant cells are indicated by white arrows (G, I, and K).

GFP gradually dominated the population. After the first, second, and third rounds of passage, 72%, 35%, and 4% of the infectious viruses were GFP-positive, respectively (Fig. 4B). Next, we compared the growth kinetics of the GFP-WNV (passage 0) with that of wild-type virus in Vero cells. The GFP-WNV replicated slower with a lower peak titer than those of the wild-type virus (Fig. 4C). Overall, the results suggest that the plaque-purified GFP-WNV is not stable in maintaining the reporter gene. However, unpassaged plaque-purified GFP-WNV is homogeneous and could be used for detection of initial WNV infection.

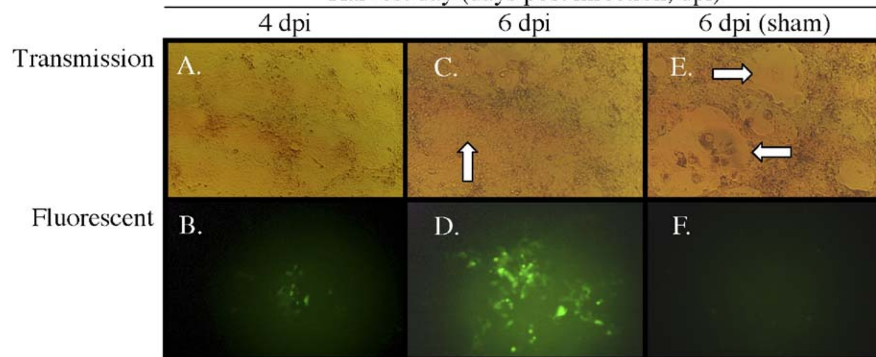
Discussion

The findings of this study suggested that differentiated syncytiotrophoblasts of the maturing placenta are a barrier to infection of mouse fetuses by WNV. The percentage of fetuses becoming infected with WNV was greater if dams were infected before, but not after 10.5 dpc. At this gestational time, the placental barrier between maternal and fetal blood is established in mice and consists of one layer of cellular trophoblast and two layers of differentiated syncytiotrophoblast possessing tight cellular structure (Georgiades et al., 2002). In this study, differentiated TGC in culture were resistant to infection with WNV, where no virus was detected up to 6 dpi when TGC were infected with WNV. Pre-implantation embryos were susceptible to viral infection after the formation of the trophectoderm at the blastocyst stage, which suggests that viral infection of embryos may be dependent on the presence of susceptible trophoblast cells. This indicates a possible mechanism for infection of the fetus with WNV through replicative trophoblast cells that are resistant after differentiation and formation of the placental barrier. Many more studies are available that demonstrate the dependence of viral infection on cellular differentiation. Human choriocarcinoma cells are susceptible to transduction with replication-incompetent adenovirus and herpes simplex virus, unless the cells are chemically differentiated (Parry et al., 1997). This loss of recombinant adenovirus and herpesvirus transduction is likely due to the downregulation of the Coxsackie adenovirus receptor during differentiation (Koi et al., 2001a, 2001b) and reduction in viral uptake, respectively. Conversely, adeno-associated virus, a parvovirus, has a higher transduction rate in differentiated cells as compared with undifferentiated cells (Koi et al., 2001a, 2001b).

Trophoblast cell differentiation in the placenta is an ongoing process. Undifferentiated placental cells are present in the placenta throughout gestation, so, during the formation of the

Trophoblast stem (TS) cells differentiated into trophoblast giant cells after infection

Harvest day (days post infection, dpi)



TS cells infected and maintained as proliferative TS cells (non-differentiated)

Harvest day (dpi)

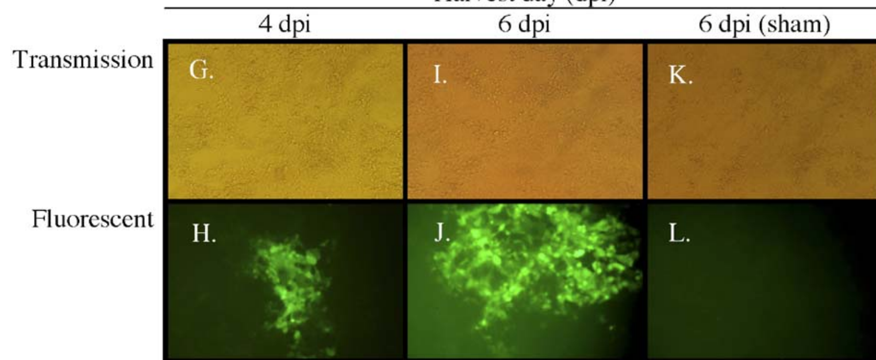


Fig. 3. Fluorescent and light images of trophoblast stem (TS) cells infected with a West Nile construct expressing green fluorescent protein (WNV-GFP). One group of cells (A–F) was differentiated to trophoblast giant cells (TGC), and another group (G–L) was maintained in a replicative state. Faint fluorescence was observed in TGC cultures (B) starting 4 days post infection (dpi), which increased in intensity on 6 dpi (D). Fluorescent cells were also observed in replicative TS cells on 4 (H) and 6 dpi (J). No GFP was observed in sham control cells (F and L). TGC were present in cultures of differentiated TS cells (D and F white arrows).

placental barrier, there is a possibility for TS cells or other progenitor trophoblast cell types to become infected and then differentiate into syncytiotrophoblast in vivo. We observed that if cultured TS cells were infected and then differentiation was induced 2 dpi, the resulting differentiated TGC had high virus-induced cell damage as compared to replicative TS cells. Differentiation of infected trophoblast stem cells into syncytiotrophoblast cells in vivo and subsequent cytopathogenesis of these cells could account for placental dysfunction and spontaneous abortion observed in potential viral infections of the placenta. Similarly, infection of extravillous cytotrophoblast cells with adenovirus resulted in apoptosis of the cells in vitro, which is a possible explanation for placental dysfunction observed in in vivo adenovirus infection (Koi et al., 2001a, 2001b). Another example of viral-induced pathogenesis of differentiated placental cells is HCMV infection of villious syncytiotrophoblast, which results in an upregulation of intercellular adhesion molecule ICAM-1 that causes blood monocytes to bind to these cells and induce apoptosis (Chan et al., 2004). Increased apoptosis within villous trophoblast cells, resulting in placental failure and fetal death, was correlated with parvovirus B-19 infection and presents further evidence of differentiated trophoblast destruction as a result virus infection as a cause for placental failure (Jordan and Butchko, 2002). We also observed fetal death if dams were infected before 10.5 dpc and not treated with WNV-reactive antibody (Julander et al., 2005).

Although the placenta did reduce the transfer of WNV to fetuses after 10.5 dpc, the placenta had high viral titers relative to all other tissues assayed, regardless of the gestational state at the time of infection, indicating the presence of susceptible cells within the mature placenta. This was significant because placental infection, even without fetal infection, can result in fetal loss or other complications of normal pregnancy and may contribute to certain congenital abnormalities (Kaplan, 1993). It will be important in future studies to double-stain for WNV antigens and cellular markers in placental tissue to identify which cells are infected in the placenta at different stages of gestation.

We hypothesize that the reduction of susceptibility of TGC may be due to a reduction of specific cellular receptors that the virus uses for entry into the trophoblast cell. The expression of cell surface receptors for herpes simplex virus-1 is reduced in third term villous syncytiotrophoblast, which prevents infection of these cells during late gestation and constitutes a barrier to herpes simplex virus-1 infection of the fetus (Koi et al., 2002). The coxsackie and adenovirus receptor is present on extravillous cytotrophoblast cells, which are susceptible to infection, but once the cytotrophoblast cells differentiate into syncytiotrophoblast cells, the receptor is not expressed and the

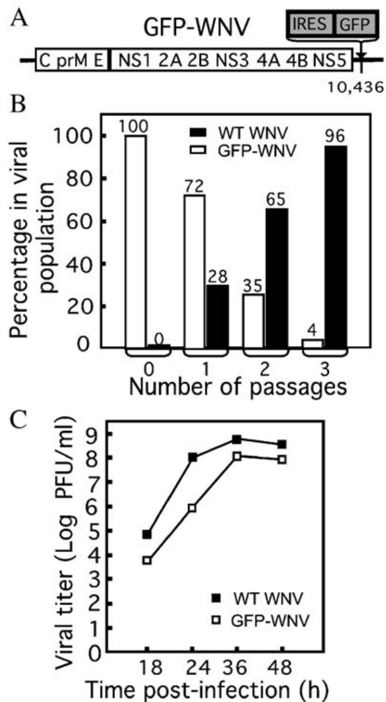


Fig. 4. A full-length WNV containing a GFP reporter (GFP-WNV). (A) GFP gene driven by an EMCV IRES was engineered at nucleotide position 10,436 within the 3′UTR of WNV genome. (B) A GFP-WNV stock (designated as passage 0, prepared by five rounds of plaque purification) was continuously passaged in Vero cells (MOI 0.1) for three rounds. At 48 h post-infection for each round, an immuno-plaque was performed to quantify GFP-positive viruses, using mouse monoclonal antibody against GFP and anti-mouse antibody conjugated with horseradish peroxidase as primary and secondary antibodies, respectively. Standard plaques assays were used to quantify total infectious WNV. The percentage of GFP-WNV and wild-type WNV in viral population was presented for each passage. (C) Growth kinetics was compared between GFP-WNV and wild-type WNV. Vero cells were infected with indicated viruses at MOI of 2. Viral production was quantified at various time points post-infection by standard plaque assays.

syncytiotrophoblast cells are not susceptible to infection (Koi et al., 2001a, 2001b). Cytomegalovirus can infect both cytotrophoblast-like or syncytiotrophoblast-like cells in vitro, indicating that differentiated cells of the functional placenta cannot always serve as a protective barrier to viral infection (Chan et al., 2004). It was anticipated that infection of trophoblast cells may be due to the presence of integrin αvβ3 on the cell surface because of the several lines of evidence that WNV uses integrin αvβ3 as a receptor for attachment and entry on Vero or other cell types (Chu and Ng, 2004). To address this hypothesis, we stained for integrin αvβ3 on the surface of replicative and differentiated trophoblast cell types. Similar levels of integrin αvβ3 were present on both replicative and differentiated trophoblast cells, and blocking with antibodies to this integrin did not reduce viral attachment and entry as seen in Vero cells (data not shown).

It is possible that the TGC will not support WNV replication because they are not actively dividing, but this is likely not the case. When TS cells were infected prior to differentiation into TGC, this resulted in differentiated placental cell types that supported the replication of the virus, and GFP expression was observed in these cells. Furthermore, cells that do not actively divide, such as neuronal cells, are susceptible to WNV infection (Ceccaldi et al., 2004; Hunsperger and Roehrig, 2005).

In summary, the involvement of trophoblast cell differentiation and placental development as a barrier to WNV infection extends our basic understanding of WNV biology and may provide information to help manage WNV infection of pregnant human patients.

Materials and methods

Animals

FVB/N mice were used (Charles River Laboratories, Wilmington, MA) because they are commonly employed to obtain embryos for micromanipulation procedures. Moreover, the FVB/N mice were susceptible to WNV disease and mortality. WNV-infected pregnant females had a 100% mortality rate and generally died prior to parturition (Julander et al., 2005). Animals were housed in the Biosafety Level 3 (BL-3) area of the AAALAC-accredited Laboratory Animal Research Center (LARC) at Utah State University (USU). Nine to 12-week-old animals were also bred in-house, and potential pregnancy was identified according to the presence of vaginal plugs, which indicated 0.5 dpc. Animal use and care were in compliance with the USU Institutional Animal Care and Use Committee.

Virus

A New York WNV crow brain stock (NY, CDC 996625, V1 D3 11/10/1999) was grown on MA-104 cells and had a titer of $10^{6.75}$ 50% cell culture infectious doses/0.1 ml ($TCID_{50}$). Virus was diluted in Minimal Essential Media (MEM) with no fetal bovine serum (FBS) for injection into timed-pregnant mice. Virus was administered subcutaneously (sc) at $10^{5.3}$ $TCID_{50}$/mouse.

A WNV construct that expresses green fluorescent protein (WNV-GFP) was used in in vitro infection studies (Fig. 4). An infectious cDNA clone of an epidemic WNV (Shi et al., 2002a, 2002b) was used to construct the WNV-GFP. The GFP reporter driven by an encephalomyocarditis virus internal ribosomal entry site (EMCV IRES) was inserted into the WNV 3′UTR at nucleotide position 10,436 (GenBank accession number AF404756). The WNV-GFP cDNA plasmid was prepared by swapping the *Spe*I–*Sac*II fragment (nt 8022 to 10,822) between the wild-type infectious clone (Shi et al., 2002a, 2002b) and a replicon clone containing the IRES-GFP insertion (Shi et al., 2002a, 2002b). The WNV-GFP RNA was in vitro transcribed and transfected into BHK cells to generate the GFP-reporting virus stock (Shi et al., 2002a, 2002b). A similar WNV containing a luciferase reporter was recently reported (Deas et al., 2005). Since such reporting WNV was shown to be unstable (Deas et al., 2005), the GFP-expressing WNV used in this study was plaque-purified to maximize its homogeneity (see details in Results). Upon infection of susceptible cells with

the WNV-GFP, GFP protein was expressed only during viral replication allowing the distinction between virus attached to cells and replicating virus.

Infectious cell culture assay

The virus titers in tissues or plasma were assayed using an infectious cell culture assay (Morrey et al., 2002) where a specific volume of tissue homogenate was added to the first tube of a series of dilution tubes. Serial dilutions were made and added to Vero cells (ATCC #CCL-81). Six to seven days later, cytopathic effect (CPE) was used to identify the end-point of infection (Sidwell and Huffman, 1971). Four replicates were used to calculate the infectious doses per gram of tissue (Reed and Muench, 1938). Tissue samples were washed thoroughly to remove contaminating virus as previously described (Julander et al., 2005).

Infection of pre-implantation embryos

Superovulation was induced in weanling female FVB/N mice by intraperitoneal injection with 5 international units (IU) pregnant mare's serum gonadotropin (PMSG) (Sioux Biochemical, Inc.) followed 42–48 h by an injection with 6 IU human chorionic gonadotropin (hCG) (Calbiochem). After injection with hCG, mice were set with males and checked for vaginal plugs the following morning. Embryos were harvested between 2- and 4-cell stage on 1.5 dpc. The oviduct was flushed with M2 medium (Sigma-Aldrich, Inc.) containing 3 mg/ml bovine serum albumin. The zona pelucida was removed from half of the embryos using acidic Tyrode's solution. Three replicate dishes containing 5 zona-intact and 5 zona-free embryos were placed together in a well of a 96-well plate and cultured in M16 media (Sigma-Aldrich, Inc.). Embryos were infected with 10^6 $TCID_{50}$/well WNV-GFP on 1.5 and 3.5 dpc. Infected embryos were fixed 20–30 min with 4% paraformaldehyde (Ted Pella, Inc.) 2 or 4 days after infection. After fixation, cells were observed using confocal microscopy.

Cultivation and infection of trophoblast stem cells and giant cells

Trophoblast stem (TS) cells were maintained as previously reported (Tanaka et al., 1998). Fibroblast conditioned media (FCM) containing 25 ng/ml fibroblast growth factor-4 (FGF-4) (Sigma-Aldrich, Inc.) and 1 μg/ml heparin (Sigma-Aldrich, Inc.) was used to maintain the TS cells in their replicative state. Once FGF-4, heparin, and FCM were removed from the media, cells will terminally differentiate into trophoblast giant cells (TGC) and other trophoblast cell types. Differentiated TGC were easily distinguished from other trophoblast cell types by the presence of polyploid nuclei and their large size. Cells were plated in 6- or 12-well plates with and without glass inserts. Two milliliters of media were added to each well, and cells were incubated at 37 °C for up to 6 days. Media was changed every 2 days.

WNV-GFP was added to each well containing 500 μl of media and incubated at 37 °C on a rocker for 30 min. To observe fluorescence, cells on glass slides were washed twice with PBS and then fixed with 4% paraformaldehyde for 20–30 min. Cells were washed 3 times with PBS and then mounted to a coverslip with Fluoromount G mounting media (Electron Microscopy Sciences, Hatfield, PA) or observed directly in the bottom of the wells of the culture plate.

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