

Coxsackievirus Preferentially Replicates and Induces Cytopathic Effects in Undifferentiated Neural Progenitor Cells^V

Ginger Tsueng,¹ Jenna M. Tabor-Godwin,¹ Aparajita Gopal,¹ Chelsea M. Ruller,¹ Steven Deline,¹ Naili An,¹ Ricardo F. Frausto,² Richard Milner,³ Stephen J. Crocker,^{2†} J. Lindsay Whitton,² and Ralph Feuer^{1*}

Cell and Molecular Biology Joint Doctoral Program, Department of Biology, San Diego State University, San Diego, California 92182-4614¹; Department of Immunology and Microbial Science, SP30-2110, The Scripps Research Institute, La Jolla, California 92037²; and Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla California 92037³

Received 28 October 2010/Accepted 25 March 2011

Enteroviruses, including coxsackieviruses, exhibit significant tropism for the central nervous system, and these viruses are commonly associated with viral meningitis and encephalitis. Previously, we described the ability of coxsackievirus B3 (CVB3) to infect proliferating neuronal progenitor cells located in the neonatal subventricular zone and persist in the adult murine central nervous system (CNS). Here, we demonstrate that cultured murine neurospheres, which comprise neural stem cells and their progeny at different stages of development, were highly susceptible to CVB3 infection. Neurospheres, or neural progenitor and stem cells (NPSCs), isolated from neonatal C57BL/6 mice, supported high levels of infectious virus production and high viral protein expression levels following infection with a recombinant CVB3 expressing enhanced green fluorescent protein (eGFP) protein. Similarly, NPSCs isolated from neonatal actin-promoter-GFP transgenic mice (actin-GFP NPSCs) were highly susceptible to infection with a recombinant CVB3 expressing DsRed (*Discosoma* sp. red fluorescent protein). Both nestin-positive and NG2⁺ progenitor cells within neurospheres were shown to preferentially express high levels of viral protein as soon as 24 h postinfection (p.i.). By day 3 p.i., viral protein expression and viral titers increased dramatically in NPSCs with resultant cytopathic effects (CPE) and eventual cell death. In contrast, reduced viral replication, lower levels of CPE, and diminished viral protein expression levels were observed in NPSCs differentiated for 5 or 16 days in the presence of fetal bovine serum (FBS). Despite the presence of CPE and high levels of cell death following early CVB3 infection, surviving neurospheres were readily observed and continued to express detectable levels of viral protein as long as 37 days after initial infection. Also, CVB3 infection of actin-GFP NPSCs increased the percentage of cells expressing neuronal class III β-tubulin following their differentiation in the presence of FBS. These results suggest that neural stem cells may be preferentially targeted by CVB3 and that neurogenic regions of the CNS may support persistent viral replication in the surviving host. In addition, normal progenitor cell differentiation may be altered in the host following infection.

Nonpolio enterovirus infections are thought to be directly responsible for a majority of clinical cases of viral meningitis and encephalitis in the United States every year. An estimated 10 to 15 million symptomatic enterovirus infections every year may account for up to 75,000 cases of meningitis hospitalizations in the United States alone (35). In particular, coxsackievirus B (CVB) and enterovirus 71 have been routinely identified in patients suffering from viral meningitis. Other serious central nervous system (CNS) diseases may result following enterovirus infection, including acute disseminating myelitis (12) and acute transverse myelitis (20). Despite the significance of these viruses in human disease, much remains to be determined regarding their neurotropism, immune activation

following infection, and potential long-lasting effects on the central nervous system in the surviving host.

We previously described a neonatal mouse model of coxsackievirus B3 (CVB3) infection whereby nestin-positive neural stem cells and myeloid cells were identified as the primary target cells during early infection (15, 16, 37). Eventually, many cells infected with CVB3 underwent apoptosis (15). However, host survival was commonly observed in parallel with detectable levels of viral RNA in the adult CNS for at least 90 days postinfection (p.i.). The ability of CVB3 to persist in other organs, in particular, the heart, has been well documented (6, 25) and may involve genetic alterations in the virus which may limit replication and cytopathic effects (CPE) in the host cell (23, 24, 36). We hypothesized that the continued presence of viral RNA and/or viral gene products may affect normal neural stem cell migration and/or differentiation in the developing CNS.

Relatively little is known about the susceptibility of neural stem cells to neurotropic viral infections. Surprisingly, neural stem cells residing in the CNS remain active into adulthood, replenishing neurons within the olfactory bulb and dentate gyrus (3, 19). As these neural stem cells give rise to mature

* Corresponding author. Mailing address: Cell and Molecular Biology Joint Doctoral Program, Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-4614. Phone: (619) 594-7377. Fax: (619) 594-0777. E-mail: rfeuer@sciences.sdsu.edu.

† Present address: Department of Neuroscience, Faculty of Medicine, University of Connecticut Health Center, Farmington, CT 06030-3401.

Published ahead of print on 6 April 2011.

neurons, their proliferative and activation status may render them attractive targets for neurotropic viruses. Also, the migratory nature of immature neuroblasts may assist in virus dissemination within the brain following infection of progenitor or stem cells (15). Recently, both human cytomegalovirus (7, 27), HIV-1 (31, 34), and Japanese encephalitis virus (9, 10) have been shown to target neural stem cells and may influence stem cell function (26, 29, 30) and immunogenicity (11).

We wished to investigate the ability of CVB3 to infect neural stem cells grown in culture in order to more clearly evaluate the consequences of CVB3 infection on stem cell survival and dysfunction in a less complex environment. Neural progenitor and stem cells (NPSCs) isolated from the brains of 1-day-old mice form neurosphere aggregates which can be passaged indefinitely in culture. NPSCs or their differentiated counterparts were infected with recombinant coxsackieviruses expressing either enhanced green fluorescent protein (eGFP-CVB3) or *Discosoma* sp. red fluorescent protein (DsRed-CVB3) and inspected for virus production and alterations in stem cell function. Also, the levels of virus replication and virus protein expression in NPSCs were compared to those of their differentiated counterparts. Our results suggest that virus production and protein expression levels were robust in undifferentiated neurospheres, yet differentiated cells appeared to be refractory to infection and virus protein expression.

Surprisingly, some NPSCs survived infection and supported a “carrier state” infection, provided that cultures were regularly replenished with fresh complete NPSC medium. We hypothesize that CVB3 may persist in a similar fashion *in vivo* within a quiescent subset of neural stem cells. Evaluating CVB3 infection of NPSCs in culture may help us understand factors influencing preferential viral replication in dividing progenitor cells, and these investigations may ultimately illuminate possible chronic alterations in neural progenitor cell differentiation during persistent infection within the surviving host.

MATERIALS AND METHODS

Isolation and production of a recombinant coxsackievirus. The generation of a recombinant coxsackievirus expressing eGFP has been described previously. Briefly, the CVB3 infectious clone (pH 3) (obtained from Kirk Knowlton at University of California at San Diego) was engineered to contain a unique SfiI site, which facilitates the insertion of any foreign sequence into the CVB3 genome. The generation of recombinant coxsackievirus expressing the enhanced green fluorescent protein (eGFP-CVB3) and DsRed (DsRed-CVB3) has been described previously (14, 37). Virus titrations were carried out as described previously (14). Viral stocks were prepared on HeLa RW cells maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). Viral stocks were diluted in DMEM before inoculation.

Isolation, culture, and infection of neurospheres. Mouse experimentation conformed to the requirements of the San Diego State University Animal Research Committee and the National Institutes of Health. C57BL/6 mice and actin promoter-GFP transgenic mice were obtained from the Scripps Research Institute animal facilities or Harlan Sprague Dawley (Harlan Laboratories, San Diego, CA). Breeding pairs were checked every day. As described previously, NPSCs were derived from isolated cortices of newborn mice, mechanically and enzymatically dissociated, and then plated as single-cell suspensions in complete NPSC medium consisting of DMEM/F12 medium supplemented with 2% B27 (Invitrogen), 20 ng/ml epidermal growth factor (EGF; Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF; Preprotech), 5 µg/ml heparin (Sigma), and 0.5% penicillin-streptomycin (8). Free-floating neurospheres were separated and transferred into new flasks every 2 days. Neurospheres were vigorously dissociated and resuspended in NPSC culture medium to a concentration of 10^5 cells/ml

in a T-25 flask (BD Falcon). Neurospheres were plated onto chamber slides and infected with eGFP-CVB3 at various multiplicities of infection (MOI) in NPSC medium. Alternatively, NPSCs were differentiated for 5 or 16 days in differentiation medium consisting of DMEM supplemented with 1% FBS, N1 supplement (Sigma), and 0.5% penicillin-streptomycin. Following infection, differentiated cells were continuously cultured in either differentiation medium or in NPSC medium. Supernatants were harvested over time to determine viral titers. The percentage of dead cells was determined by trypan blue staining, followed by cell counting using a hemacytometer. Neurosphere cultures were replenished with complete NPSC medium and passed every 3 days up to 37 days postinfection. Infected neurospheres were examined over time using fluorescence microscopy.

Immunofluorescence microscopy. Live NPSCs or differentiated NPSC cultures infected with eGFP-CVB3 or DsRed-CVB3 were imaged using a Zeiss Axio Observer D.1 inverted fluorescent microscope with an ApoTome Imaging System. Alternatively, infected NPSCs were fixed in 4% paraformaldehyde and washed three times in phosphate-buffered saline (PBS). Viral protein expression was determined by native eGFP (green) or DsRed (red) expression. Fixed cells were blocked with 10% normal goat serum (NGS) and immunostained using the following antibodies: nestin (catalog item PRB-315C; Covance, Inc.) at 1:1,000, neuronal class III β-tubulin (PRB-435P; Covance, Inc.) at 1:1,000, glial fibrillary acidic protein ([GFAP] G 9269; Sigma, Inc.) at 1:1,000, NG2 (AB5320; Chemicon, Inc.) at 1:500, Olig2 (ab33427; Abcam, Inc.) at 1:1,000, and myelin basic protein (MBP) at 1:1,000 (AB980; Chemicon, Inc.). Primary antibodies were diluted in 2% NGS in PBS (150 to 200 µl per slide) in a humidified chamber and incubated overnight. Slides were washed with PBS for 5 min (three times). Secondary antibodies (at 1:1,000) conjugated to Alexa-Fluor-594, Alexa-Fluor-488, or Alexa-Fluor-350 were diluted with 2% NGS in PBS (150 to 200 µl per slide) and incubated overnight. Following incubation with the secondary antibody, slides were washed with PBS for 5 min (three times). Three to five representative images of the cultures were taken for each sampling time point at multiple magnifications.

ImageJ analysis. For each fluorescent image, three fluorescent channels for each image were exported separately without color overlay. Threshold adjustments were applied to generate a black and white image using NIH ImageJ (public domain software). These black and white images were analyzed using ImageJ to quantify fluorescent signal. In each case, marker expression was normalized prior to applying a Student *t* test to determine statistical significance. In C57BL/6 NPSCs infected with eGFP-CVB3, marker expression was normalized to 4',6'-diamidino-2-phenylindole (DAPI). In actin-GFP NPSCs infected with DsRed-CVB3, marker expression was normalized to GFP.

RESULTS

CVB3 infection of primary neural progenitor and stem cells (NPSCs) in culture. We isolated and cultured primary NPSCs from both C57BL/6 and actin promoter-GFP transgenic mice in order to determine their ability to support CVB3 infection. NPSCs grown in culture formed neurospheres, which comprise both stem and progenitor cells. As expected, isolated neurospheres expressed high levels of nestin, a marker for neural stem and progenitor cells, as determined by immunofluorescence (data not shown). Neurospheres were infected at a multiplicity of infection (MOI) of 0.1 with a recombinant coxsackievirus B3 expressing eGFP (eGFP-CVB3), and viral protein expression (eGFP) was evaluated by immunofluorescence microscopy merged with Hoffman modulation contrast (Hoffman MC) over the course of 7 days (Fig. 1).

As early as 1 day postinfection (p.i.), virus protein expression was observed in a minority of cells within the neurosphere (Fig. 1A). An increase in virus protein expression was readily seen in neurospheres by day 2 p.i. (Fig. 1B). By day 4 p.i., nearly all cells within many neurospheres expressed large amounts of viral protein (Fig. 1C). In contrast, differentiated cells located near the periphery of the neurosphere which had attached to the surface of the chamber slide (most likely following the depletion of growth factors in the complete NPSC medium)

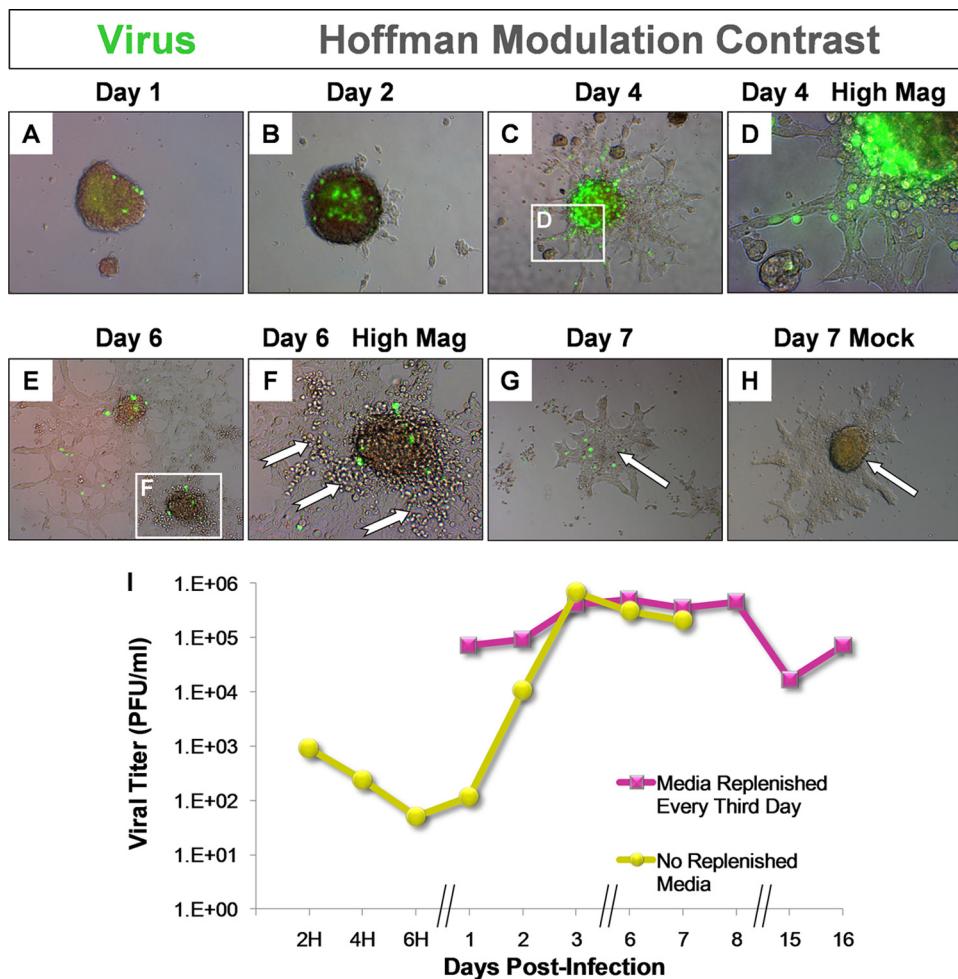


FIG. 1. Neurospheres grown in culture were highly susceptible to eGFP-CVB3 infection. NPSCs were isolated from the cortices of 1-day-old C57BL/6 mice, cultured to form neurosphere aggregates, and infected with eGFP-CVB3 (MOI of 0.1). Infected neurospheres were observed over time by fluorescence microscopy, and viral titers were determined by plaque assay. (A) Virus protein expression (green) was readily observed by day 1 p.i. (B and C) An increase in viral protein expression was seen until day 4 p.i. (D) Higher magnification of panel C showed the preferential infection of neurosphere aggregates with little to no viral protein expression in adherent cells with stretched morphology. (E) By day 6 p.i., virus protein levels were reduced, and signs of cytopathic effect (CPE) were readily observed at a higher magnification (F, notched white arrows). (G) By day 7 p.i., viral protein levels were low, many neurospheres had disappeared, and the remaining cells consisted of adherent cells (white arrow). (H) In contrast, neurospheres were readily apparent in mock-infected cultures (white arrow). (I) Viral titers increased over time and reached a maximum at day 3 p.i. (yellow). Also, infected NPSCs replenished with complete medium every 3 days supported a carrier state infection with high levels of infectious virus (pink).

failed to express appreciable levels of viral protein. Higher magnification of day 4 infected cultures illustrated the lack of viral protein expression in differentiated cells adjacent to heavily infected neurospheres (Fig. 1D). By day 6 p.i., the level of viral protein expression dropped dramatically (Fig. 1E), and cytopathic effects (CPE) were readily apparent near residual neurospheres (Fig. 1F, notched white arrows). At 7 days p.i., many infected neurospheres disappeared, presumably due to CPE (Fig. 1G, white arrow), and viral protein expression was limited to rounded cells or cellular debris while differentiated cells continued to lack viral protein expression. In contrast, unharmed neurospheres remained visible in mock-infected control cultures grown in parallel for 7 days (Fig. 1H, white arrow).

We evaluated the amount of infectious virus produced over time in these infected neurosphere cultures by plaque assay

(Fig. 1I, yellow line). Within 2 days p.i., viral titers increased dramatically in neurosphere cultures. By 3 days p.i., peak production of infectious virus was observed, which corresponded closely with viral protein expression levels, as determined by immunofluorescence microscopy. By day 6 p.i. and beyond, viral titers dropped, corresponding to the presence of CPE within the neurosphere cultures. Taken together, these results suggest that virus protein expression and viral replication were robust in stem and progenitor cells found within neurospheres; in contrast, differentiated cells adjacent to infected neurospheres appeared to be refractory to infection.

Also, NPSCs were infected with eGFP-CVB3 at a higher MOI (MOI of 100.0), replenished with complete NPSC medium every third day, and followed for infectious virus production by plaque assay for 16 days p.i. (Fig. 1I, pink line). High viral titers were observed in these NPSC cultures for up to 16

days p.i. Surprisingly, some NPSCs survived initial infection in the presence of replenished complete NPSC medium, and these infected NPSCs supported a carrier state infection characterized by a steady-state infection in which many or all of the cells became infected (18). These findings suggest the presence of a subpopulation of stem cells which may be resistant to virus-mediated CPE; a resistant primary stem cell may restore the neurosphere culture and generate additional target cells for the maintenance of virus production over time.

Preferential infection of nestin-positive and NG2⁺ cells in neurosphere cultures. Although NPSCs consist of nestin-positive stem cells, cells lacking nestin expression were also found within neurospheres. These nestin-negative cells may represent more committed progenitor cells or immature neuronal or glial cells. To determine which population of cells within a neurosphere were most susceptible to infection, NPSCs were infected with eGFP-CVB3 (MOI of 0.1), harvested 2 days later, and immunostained for nestin, as well as three additional downstream lineage markers including neuronal (neuronal class III β-tubulin) and glial cell (GFAP and NG2) markers. Neuronal class III β-tubulin is highly expressed within neuroblasts and immature neurons. GFAP is a marker commonly utilized to discriminate immature glial cells and astrocytes (28). NG2 has been used to identify oligodendrocyte precursor cells (4). A Zeiss Axio Observer with ApoTome Imaging System was utilized to detect the colocalization of infected cells and cells expressing downstream lineage molecules. The ApoTome Imaging System utilizes structured illumination technology allowing for the collection of optical sections and three-dimensional reconstruction.

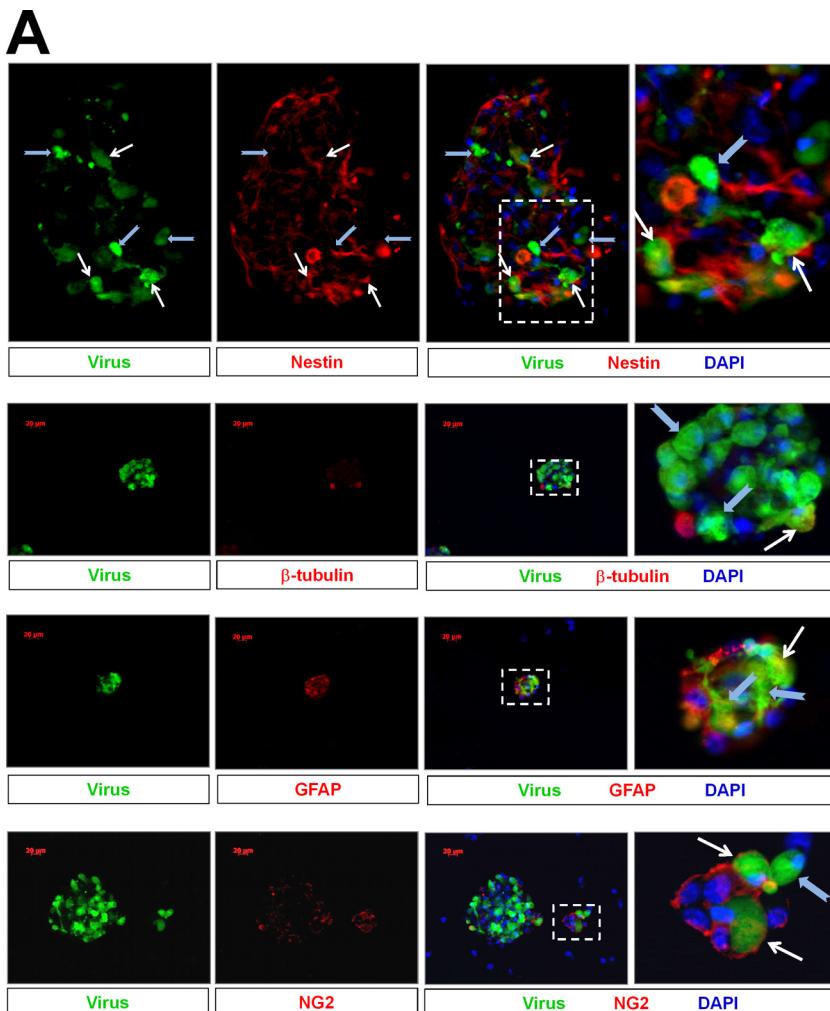
Both nestin-positive and nestin-negative NPSCs expressed high levels of viral protein (Fig. 2A, white arrow and blue arrow, respectively). Also, we detected the expression of three downstream lineage markers within infected neurospheres at 2 days p.i. (Fig. 2A, β-tubulin, GFAP, and NG2 immunostaining). Viral protein expression was observed in cells expressing all three downstream markers although infection was preferentially within NG2⁺ cells. In Fig. 2A, higher magnification of a boxed field for each marker shows infected cells either expressing (white arrows) or failing to express (blue arrows) the marker. The number of infected cells expressing each marker was quantified over 2 days (Fig. 2B). Four cell populations were analyzed, and the proportion of infected cells was found to be highest in nestin-positive cells at day 1 and day 2 p.i. (66.7% and 75%, respectively). Also, an increasing percentage of NG2⁺ cells expressed detectable levels of viral protein at day 1 and day 2 p.i. (40% and 70.5%, respectively). The least percentage of infected cells was observed in β-tubulin-positive cells (9.1% and 14.9% at day 1 and day 2 p.i., respectively) and GFAP-positive (GFAP⁺) cells (10.4% and 14.8% at day 1 and day 2 p.i., respectively) although the percentage of double-positive cells increased for both populations over the course of 2 days. These results suggest that CVB3 may be preferentially infecting progenitor cells which follow the oligodendrocyte pathway or, alternatively, that the differentiation pathway of infected stem cells may be preferentially shifted toward the oligodendrocyte lineage.

Virus replication and cell death in actin promoter-GFP neurospheres infected with DsRed-CVB3. We tested the ability of an additional recombinant coxsackievirus expressing DsRed

(DsRed-CVB3) to infect an independent isolation of neurospheres from actin promoter-GFP transgenic mice (Fig. 3). Also, the infection of actin promoter-GFP neurospheres (actin-GFP NPSCs) was carried out at two multiplicities of infection (0.1 and 10.0) and followed over 7 days in culture. The eventual fate of infected neural stem cells surviving acute infection and followed via the adoptive transfer into the CNS will be of great interest in future studies involving actin-GFP NPSCs. Actin-GFP NPSCs were highly susceptible to infection with DsRed-CVB3 and supported high levels of viral protein expression (DsRed) at an MOI of 0.1 (Fig. 3A to E) and an MOI of 10.0 (Fig. 3F to J). At an MOI of 0.1, viral infection of actin-GFP NPSCs led to CPE at day 3 p.i. (Fig. 3C and L, white arrows). We observed accelerated CPE at the higher MOI in cultures infected with DsRed-CVB3 at day 2 p.i. (Fig. 3G and K, white arrows). The high level of virus protein expression (red) in actin-GFP NPSCs was represented by single-channel images (Fig. 3G, inset, and M). In contrast, no CPE was observed in mock-infected actin-GFP NPSCs at any time point (Fig. 3N). By 7 days p.i., only cellular debris remained for the majority of actin-GFP NPSCs infected at either a high or low MOI (Fig. 3E and J). High viral titers were observed over 7 days in actin-GFP NPSCs infected at either a high or low MOI (Fig. 3O). As expected, viral titers rose more quickly in actin-GFP NPSCs infected at a higher MOI (Fig. 3O).

We inspected the degree of cell death in NPSCs following CVB3 infection over the course of 3 days p.i. (Fig. 4). NPSCs isolated from C57BL/6 mice were infected with eGFP-CVB3 at two multiplicities of infection (MOI of 0.1 or 10.0), and viral protein expression was followed by fluorescence microscopy (Fig. 4B and C). Alternatively, actin-GFP NPSCs were infected with DsRed-CVB3 at two multiplicities of infection (MOI of 0.1 and 10.0) and compared directly to neurospheres infected with eGFP-CVB3 (Fig. 4E and Fig. F). Both recombinant coxsackieviruses induced rapid CPE in NPSCs at the greater MOI (MOI of 10.0) by day 3 p.i. (Fig. 4C and F, white arrows). Conversely, the greater level of CPE observed at the higher MOI may be due to more cells infected initially and yet more readily detected by our trypan blue staining protocol. By trypan blue staining, the percentage of dead cells was determined over time for C57BL/6 NPSCs infected with eGFP-CVB3 (Fig. 4G) and for actin-GFP NPSCs infected with DsRed-CVB3 (Fig. 4H). An increase in the percentage of dead cells was observed over 3 days with both recombinant viruses. Also, NPSCs infected at a greater MOI (MOI of 10.0) showed accelerated and higher levels of cell death by trypan blue staining at day 3 p.i., a time point when CPE was observed in cultures by fluorescence microscopy. These results demonstrate that neurospheres from two independent isolation procedures were similarly susceptible to infection and CPE using two recombinant coxsackieviruses.

Reduced virus replication in NPSCs differentiated with FBS. We inspected in greater detail the susceptibility of differentiated cells derived from NPSCs to CVB3. NPSCs were cultured in the presence of FBS in order to increase surface attachment and induce differentiation (Fig. 5). Prior to the addition of FBS, NPSCs expressed high levels of nestin; however, in the presence of FBS, neurosphere aggregates began to attach to the surface, reduced their expression of nestin, and expressed high levels of neuronal class III β-tubulin (Fig. 5A).



B

	Antibody	Pos. Cells	Neg. Cells	Total	% Positive
Day 1 PI	Nestin	10	5	15	66.7
	β-tubulin	4	40	44	9.1
	GFAP	10	86	96	10.4
	NG2	12	18	30	40.0
Day 2 PI	Nestin	36	12	48	75.0
	β-tubulin	7	40	47	14.9
	GFAP	9	52	61	14.8
	NG2	31	13	44	70.5

FIG. 2. CVB3 preferentially infected nestin-positive and NG2⁺ cells in neurospheres. NPSCs were infected with eGFP-CVB3, harvested at day 2 p.i., and stained for four cell markers (nestin, neuronal class III β-tubulin, GFAP, and NG2). Structured illumination immunofluorescence microscopy was carried out with a Zeiss Axio Observer with ApoTome Imaging System. (A) Both nestin-positive (white arrows) and nestin-negative cells (blue arrows) were susceptible to infection within neurosphere aggregates. Higher magnification of the boxed field showed both nestin-positive and nestin-negative infected cells (white arrows and blue arrows, respectively). Also, infected cells expressed cell lineage markers, including β-tubulin, GFAP, and NG2 cell markers. Higher magnification of the boxed field for each cell lineage marker showed both marker-positive (white arrows) and marker-negative (blue arrows) infected cells. (B) The percentage of infected cells expressing each marker at day 1 and day 2 p.i. was quantified and represented in a table form. Relatively high percentages of nestin-positive and NG2⁺ cells were infected with eGFP-CVB3 at day 1 and day 2 p.i. Lower percentages of infection were seen in cells expressing β-tubulin and GFAP at day 1 and day 2 p.i. Pos, positive; Neg, negative.

NPSCs were grown in the presence of FBS for 5 or 16 days and subsequently cultured with complete NPSC medium lacking FBS (Fig. 5B). NPSCs cultured in the presence of FBS for 16 days appeared to be in a more differentiated state and attached

more robustly to the culture surface than NPSCs cultured with FBS for only 5 days. Surprisingly, NPSCs differentiated for 5 days in the presence of FBS and replenished with NPSC medium lacking FBS showed a progressive return to spherical

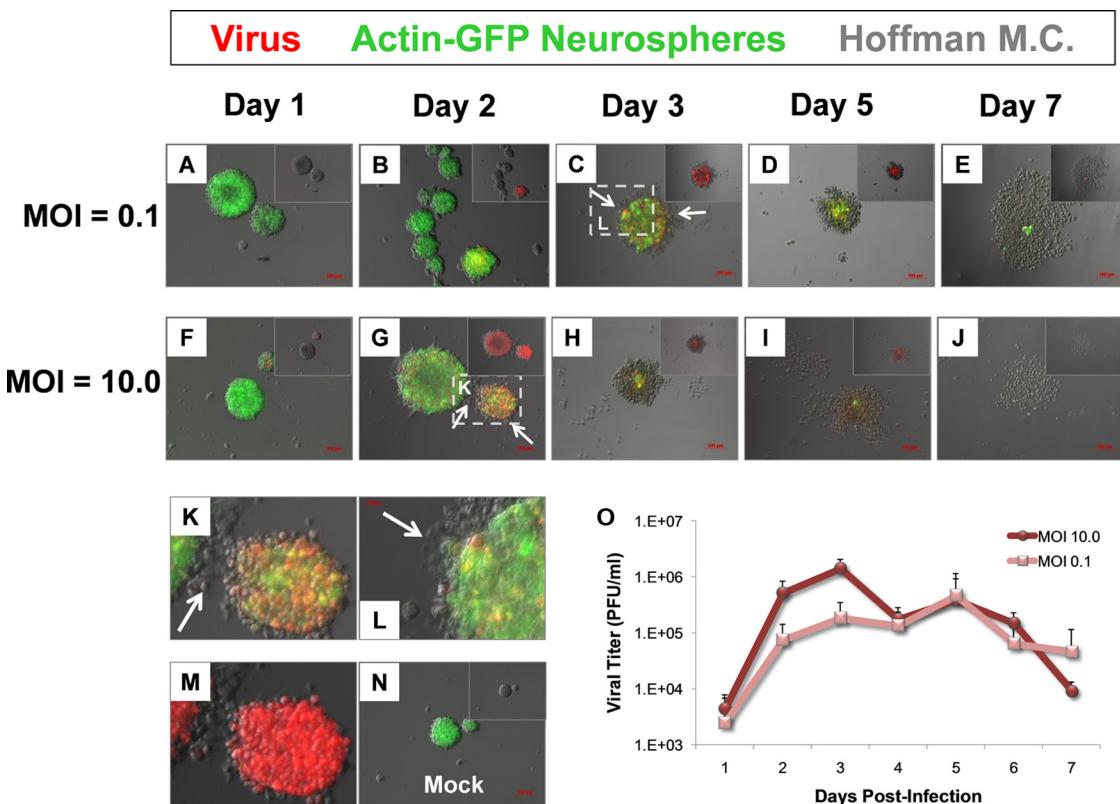


FIG. 3. Virus replication and cytopathic effects in neurospheres isolated from actin promoter-GFP transgenic mice and infected with DsRed-CVB3. Actin promoter-GFP (actin-GFP) NPSCs were isolated from the cortices of 1-day-old actin promoter-GFP transgenic mice, cultured to form neurosphere aggregates, and infected with DsRed-CVB3 (MOI of 0.1 and 10.0). Infected actin-GFP NPSCs were observed over 7 days by fluorescence microscopy, and viral titers were determined by plaque assay. (A to E) actin-GFP NPSCs infected at the lower MOI (MOI of 0.1) expressed high levels of virus protein in the majority of neurospheres (red in inset images without green signal) as soon as day 3 p.i. (white arrows). Cytopathic effects were seen at day 5 p.i. (F to J) Accelerated virus protein expression (red in inset images without green signal) at day 2 p.i. (white arrows) and CPE (at day 3 p.i.) were seen in actin-GFP NPSCs infected with the higher MOI (MOI of 10.0). (K and M) Higher magnification of panel G and a single-channel image (DsRed) revealed the relatively high level of virus protein expression and the presence of dying cells (white arrow) at day 2 p.i. in actin-GFP NPSCs infected at the higher MOI. (L) Similarly, higher magnification of panel C showed DsRed expression and the presence of dying cells (white arrow) at day 3 p.i. in actin-GFP NPSCs infected at the lower MOI. (N) No red signal or CPE was observed in mock-infected actin-GFP NPSCs. (O) Viral titers were determined for actin-GFP NPSCs infected at a low or high MOI.

neurosphere morphology at day 3 and day 7 posttreatment. Similarly, NPSCs differentiated for 16 days in the presence of FBS and replenished with NPSC medium lacking FBS also showed substantial morphological changes after 7 days in NPSC medium, suggesting “dedifferentiation” to neurosphere aggregates.

We inspected the expression levels of nestin, GFAP, neuronal class III β -tubulin (β -tubulin), and MBP in NPSCs differentiated for 5 or 16 days in the presence of FBS and replenished with complete NPSC medium lacking FBS or cultured in the presence of FBS (differentiation medium) (Fig. 5C). As expected, NPSCs treated for 5 or 16 days with FBS and cultured in differentiation medium expressed high levels of GFAP, β -tubulin, and MBP. Although nestin continued to be expressed in 5-day-differentiated cultures in differentiation medium, the cellular distribution of signal was altered (diffuse rather than filamentous). In contrast, 16-day-differentiated cultures in differentiation medium failed to express detectable levels of nestin, suggesting that these cells were well differentiated (Fig. 5C, white arrows). Paralleling the morphological results, 16-day-differentiated cultures in NPSC medium were induced to express moderate to high levels of nestin, suggest-

ing dedifferentiation, similar to what has been described previously (2, 21).

Five-day-differentiated or 16-day-differentiated NPSC cells were infected with eGFP-CVB3 and cultured either in NPSC medium or differentiation medium. Virus protein expression levels were evaluated by fluorescence microscopy up to 10 days following infection (Fig. 6). As determined previously, undifferentiated neurospheres cultured in NPSC medium and infected with eGFP-CVB3 supported robust levels of virus protein expression and CPE although surviving neurospheres were observed at day 7 and day 10 p.i. (Fig. 6, indicated by white arrows in the first column). The least amount of virus protein expression was observed in 16-day-differentiated NPSCs continuously cultured with FBS (Fig. 6, last column) although detectable levels of virus protein expression were eventually observed at day 10 p.i. within attached cells with stretched morphology. Also 5-day-differentiated NPSCs cultured continuously with FBS (Fig. 6, fourth column) expressed relatively low levels of virus protein. Little to no CPE was observed in either of these differentiated cultures continuously cultured with FBS.

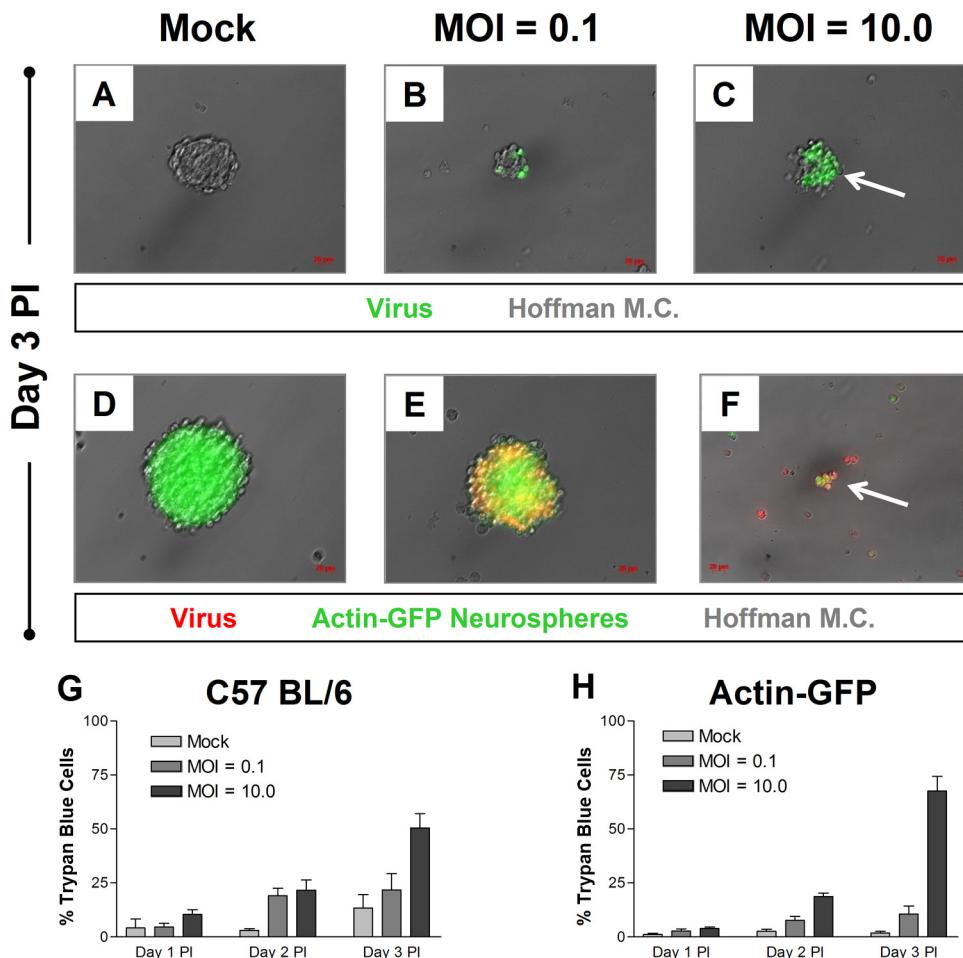


FIG. 4. Quantification of virus-induced cell death over time in neurospheres following infection with two recombinant coxsackieviruses. C57BL/6 NPSCs or actin-GFP NPSCs were mock-infected or infected with recombinant CVB3s (eGFP-CVB3 and DsRed-CVB3) at two MOI, observed by fluorescence microscopy, and stained for trypan blue at day 3 p.i. (A to C) Greater levels of virus protein (green) and accelerated CPE were observed in NPSCs infected with eGFP-CVB3 at the higher MOI (white arrow). (D to F) Similarly, greater levels of virus protein (red) and accelerated CPE were observed in actin-GFP NPSCs infected with DsRed-CVB3 at a higher MOI (white arrow). (G and H) The percentage of dead (trypan blue-positive) cells was determined in infected C57BL/6 and actin-GFP NPSCs using a hemacytometer. A greater percentage of dead cells was observed at each time point in both NPSC cultures infected at the higher MOI.

In 16-day-differentiated NPSC cultures replenished with NPSC medium, virus protein expression was observed at day 3 and continued to day 10 p.i. in the presence of low levels of CPE. In contrast to differentiated NPSCs continuously cultured with FBS, 5-day-differentiated NPSC cultures replenished with NPSC medium supported detectable virus protein expression levels very early (day 1 p.i.). At day 3 p.i., these cultures expressed high levels of virus protein in the presence of CPE (day 3 p.i.). The attached cells with a differentiated, stretched morphology in 5-day-differentiated cultures replenished with NPSC medium eventually gave rise to neurosphere aggregates with a spherical morphology, most likely reflecting dedifferentiation in these cells following the removal of FBS. The majority of 5-day-differentiated cultures replenished with NPSC medium underwent CPE by day 7 p.i.; however, surviving neurospheres were observed in these cultures (Fig. 6, indicated by a white arrow in the second column).

The morphology of many infected cells in 5-day-differentiated cultures replenished with NPSC medium suggested infec-

tion of immature neurons with long axonal extensions expressing high levels of virus protein (Fig. 7A, white arrows). Higher magnification showed the axonal processes of an infected cell extending and connecting to uninfected neighboring cells in these cultures (Fig. 7B and C, white arrows). In some cases, two infected neuronal cells with axonal processes made contact with uninfected cells separating both infected cells, suggesting preferential infection in cells with axonal morphology (Fig. 7D). Also, the presence of cellular blebbing was occasionally seen in adherent cells infected with CVB3, suggesting ongoing apoptosis in these cultures (Fig. 7E and F, blue arrows). Despite the high degree of CPE in the 5-day-differentiated cultures replenished with NPSC medium, surviving neurospheres could be observed at day 10 and day 37 p.i., suggesting dedifferentiation of the cultures and the presence of stem cells resistant to virus-mediated CPE and capable of renewing the neurosphere cultures (Fig. 7G and H, blue arrows). A closer inspection of neurospheres from day 37 p.i. indicated the continued expression of detectable levels of virus protein at this

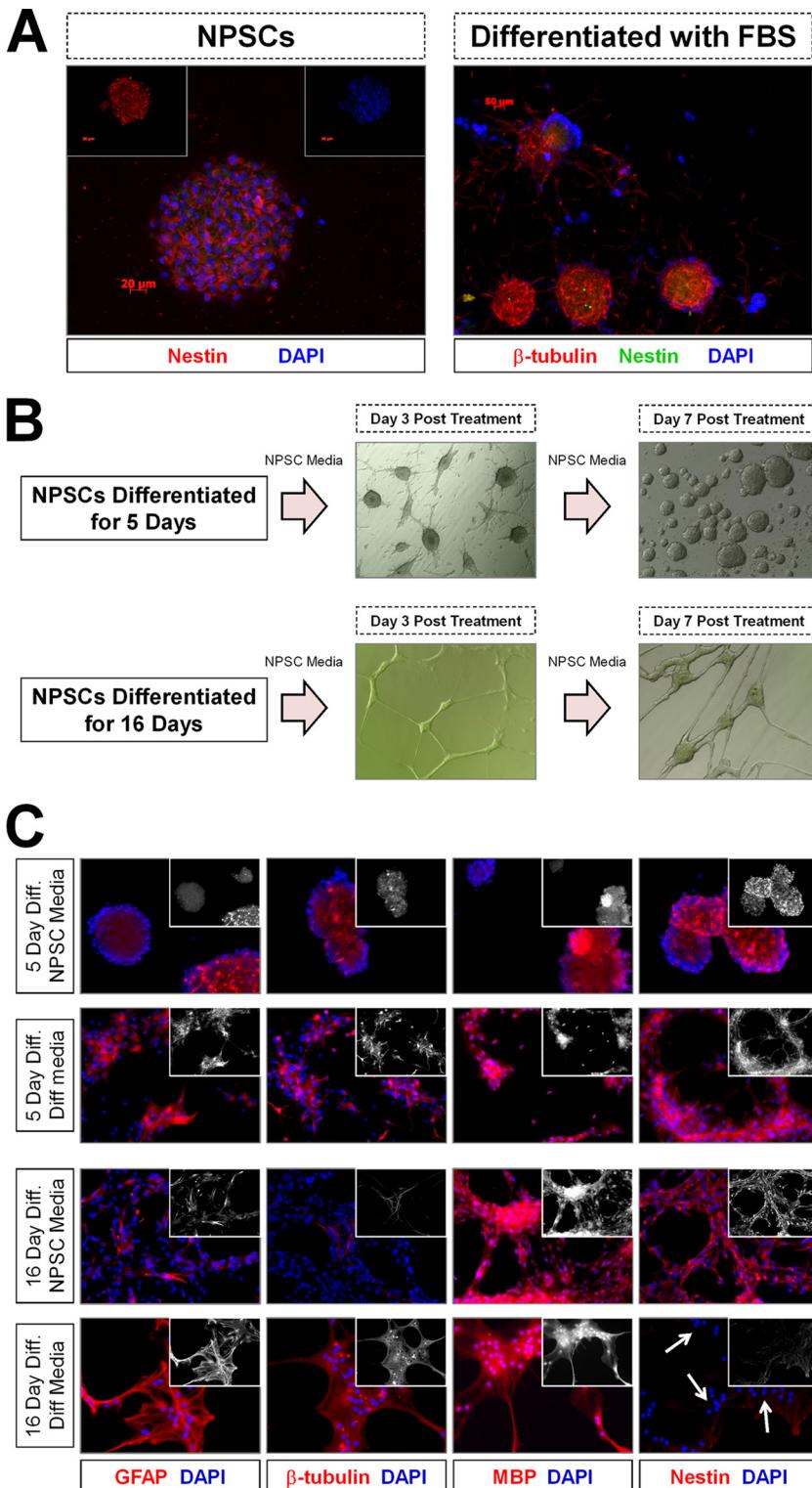


FIG. 5. Differentiation of NPSCs following treatment with fetal bovine serum. C57BL/6 NPSCs were evaluated for nestin and β -tubulin expression before and after FBS treatment. (A) NPSCs were grown in complete NPSC medium or, alternatively, were differentiated for 5 days in the presence of FBS. NPSCs or differentiated NPSCs were fixed with 4% paraformaldehyde and stained for nestin (red) or costained for both nestin (green) and β -tubulin (red). Cells were counterstained with DAPI (nuclear dye). (B) NPSCs differentiated for 5 or 16 days in the presence of FBS were placed into complete NPSC medium in the absence of FBS (NPSC medium) for an additional 3 or 7 days. Partial dedifferentiation was observed as soon as day 3 posttreatment for NPSCs differentiated for 5 days, with complete dedifferentiation at day 7 posttreatment. Also, partial dedifferentiation was observed by day 7 posttreatment for NPSCs differentiated for 16 days. (C) NPSCs were differentiated for 5 or 16 days and placed in NPSC medium or in the presence of FBS (Diff. Media) for 3 days, fixed with 2% paraformaldehyde, and immunostained for nestin, GFAP, neuronal class III β -tubulin (β -tubulin), or MBP. The 5-day- and 16-day-differentiated cells expressed high levels of nestin and reduced levels of GFAP and β -tubulin in the presence of complete NPSC medium, suggesting their dedifferentiation in these cultures. The 16-day-differentiated cells in differentiation medium expressed little or no nestin (white arrows), suggesting their differentiated status.

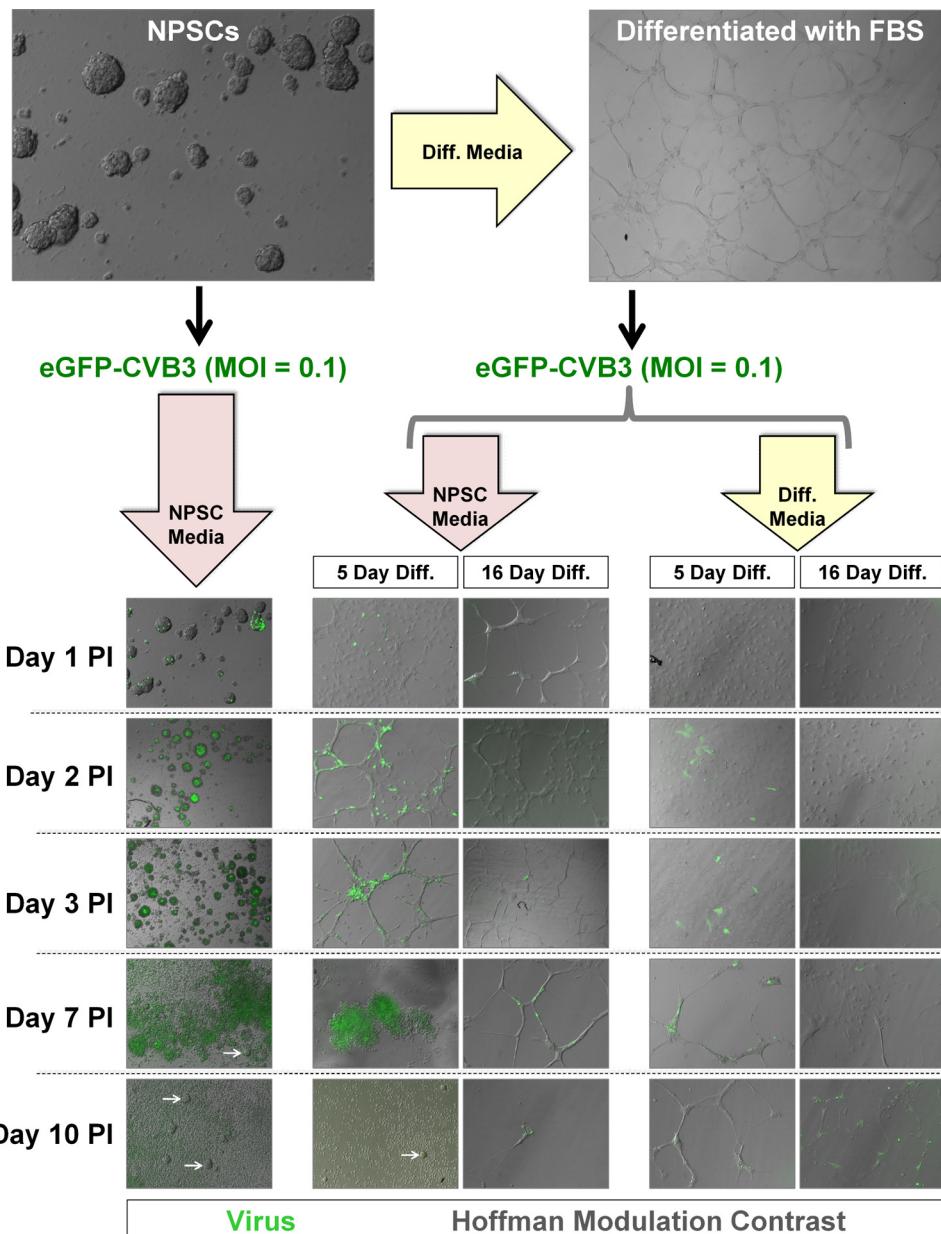


FIG. 6. Higher levels of viral protein expression and CPE were observed in NPSCs than in differentiated NPSCs. NPSCs or differentiated NPSCs (5 Day Diff. or 16 Day Diff.) were infected with eGFP-CVB3 (MOI of 0.1) and followed for virus protein expression for 10 days p.i. Following infection, NPSCs or differentiated NPSCs were cultured in complete NPSC medium lacking FBS (pink arrow). Alternatively, differentiated NPSCs infected with virus were cultured in medium containing FBS (yellow arrow). The greatest to least amount of virus protein expression and CPE were observed in the following order, from left to right: NPSCs in NPSC medium > 5-day-differentiated cells in NPSC medium > 16-day-differentiated cells in NPSC medium > 5-day-differentiated cells in differentiation medium > 16-day-differentiated cells in differentiation medium. Despite the relatively high level of CPE, surviving neurospheres continued to be observed in infected NPSCs in NPSC medium and in infected 5-day-differentiated cells in NPSC medium (white arrows).

extended time point (Fig. 7I and J, blue arrows), indicative of viral persistence in this long-term culture.

We measured viral titers in NPSCs differentiated with FBS and compared them to those of undifferentiated NPSCs up to 7 days p.i. Viral titers were evaluated in 5-day-differentiated cultures either continuously treated with FBS or replenished with NPSC medium (Fig. 7K). The highest viral titers (over 10^7 PFU/ml at day 2 p.i.) were observed in NPSCs cultured in

NPSCs medium over all time points. The 5-day-differentiated cultures replenished with NPSC medium supported lower levels of virus replication at all time points than infected NPSCs. The least amount of virus replication was observed in 5-day-differentiated cultures continuously treated with FBS. These results match the virus protein expression levels shown in Fig. 6 and suggest preferential CVB3 replication in NPSCs compared to that of their differentiated counterparts. A greater

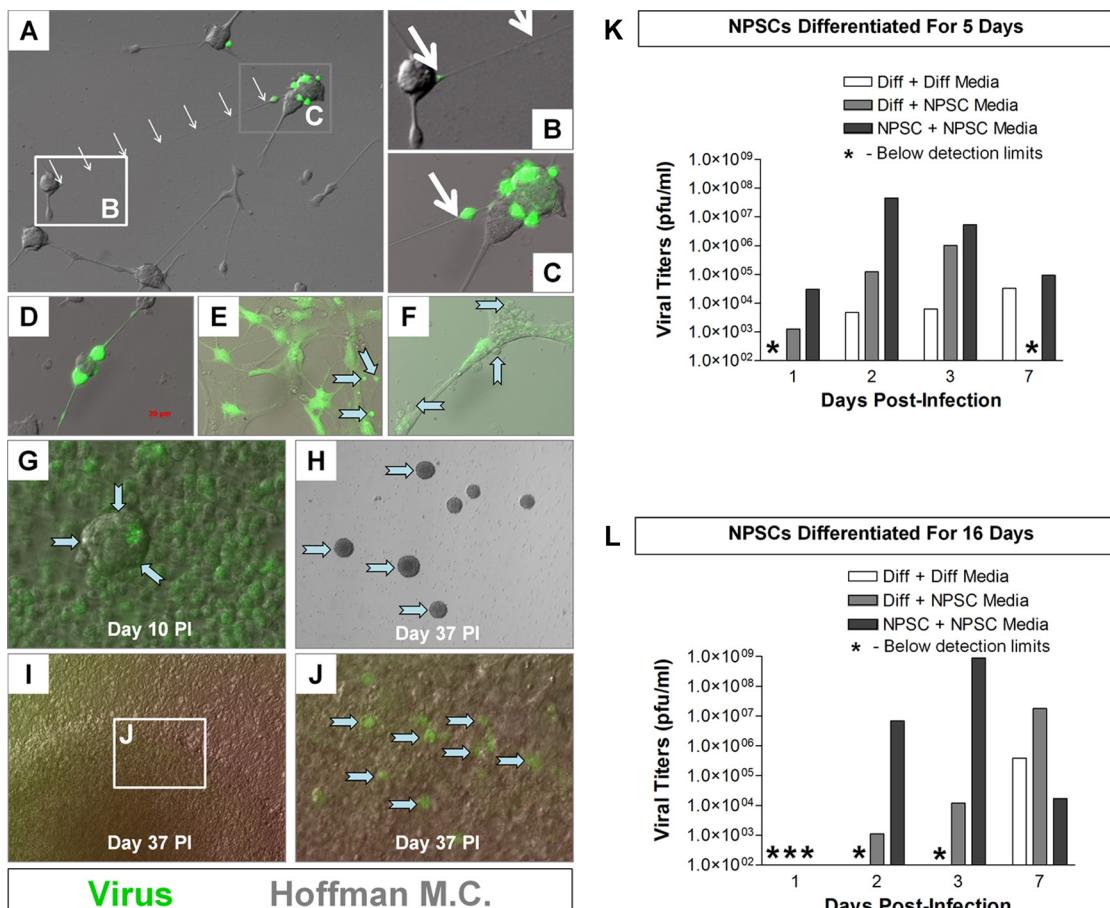
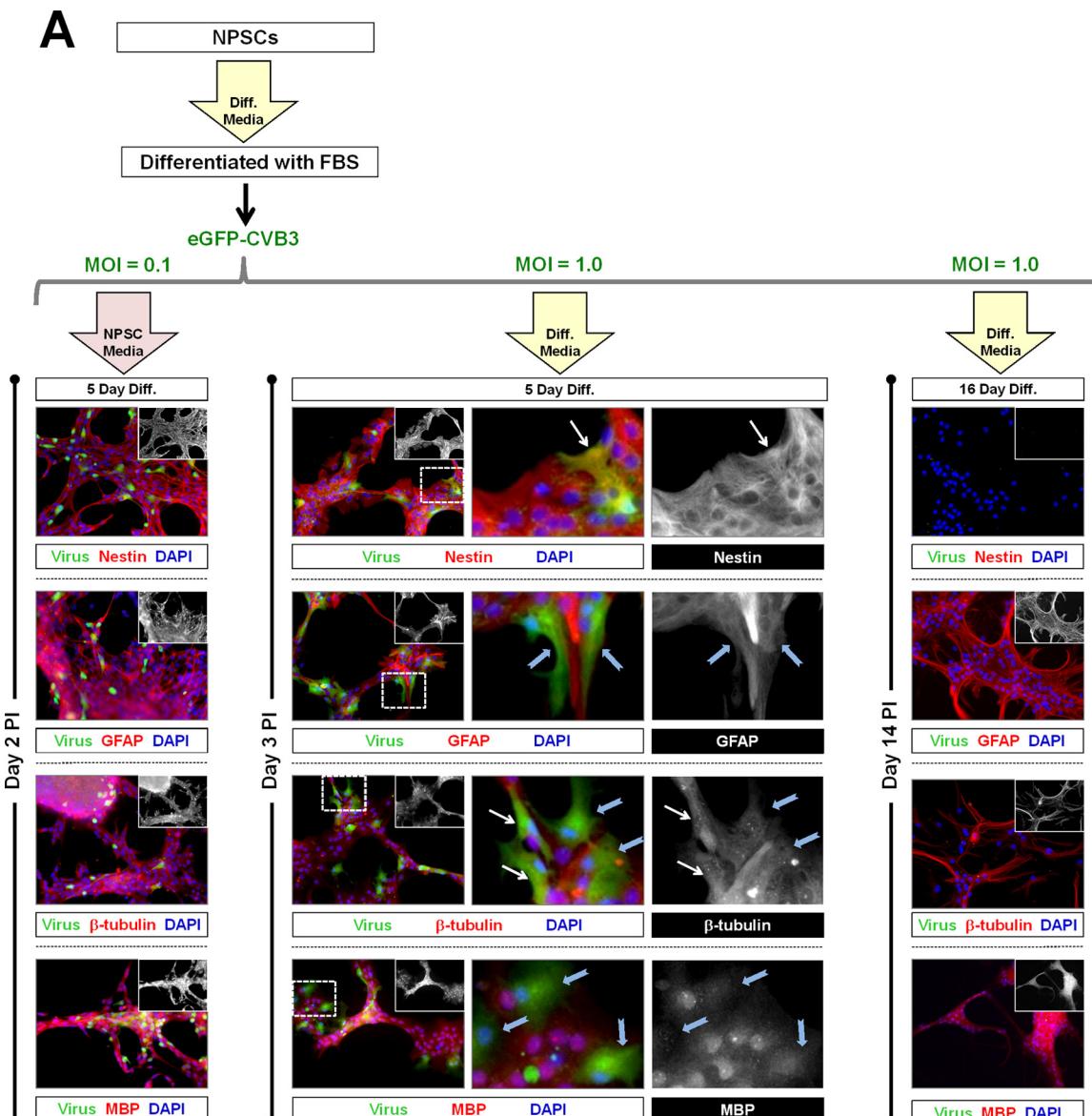


FIG. 7. Higher levels of viral titers observed in NPSCs than in differentiated NPSCs. NPSCs or differentiated NPSCs (5-day- or 16-day-differentiated [5 Day Diff. or 16 Day Diff.] cells) were infected with eGFP-CVB3 (MOI of 01) and observed by fluorescence microscopy over time. Also, viral titers were evaluated in these cultures up to 7 days p.i. Following infection, NPSCs or differentiated NPSCs were cultured in complete NPSC medium lacking FBS (NPSC medium). Alternatively, differentiated NPSCs infected with virus were cultured in medium containing FBS (Diff. Media). (A) Infected cells at day 3 p.i. with extended axonal processes were readily apparent in 5-day-differentiated cells in NPSC medium (white arrows). (B to D) Higher magnification of panel A showed the contact of infected cells with axonal processes to adjacent uninfected cells. (E and F) Cellular blebbing was seen in many differentiated NPSCs following infection, including 5-day-differentiated cells in NPSC medium at day 2 p.i. (blue arrows) and in 5-day-differentiated cells in differentiation medium at day 7 p.i. (blue arrows). Neurospheres surviving infection in NPSC medium were observed at day 10 p.i. (G) and day 37 p.i. (H). (I and J) Detectable levels of virus protein expression were observed in many surviving neurospheres at day 37 p.i. (blue arrows). (K) Viral titers were determined for NPSCs and compared to those of 5-day-differentiated NPSCs cultured in differentiation medium or NPSC medium. The cultures of 5-day-differentiated cells in differentiation medium produced the least amount of infectious virus over 7 days p.i. (L) Viral titers were determined for NPSCs and compared to those of 16-day-differentiated NPSCs cultured in differentiation medium or NPSC medium. Cultures of the 16-day-differentiated cells in differentiation medium failed to produce detectable levels of infectious virus for up to 3 days p.i.

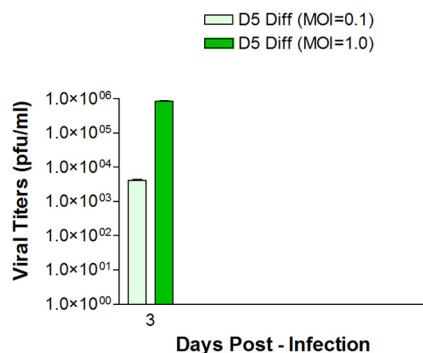
difference in viral replication was observed in 16-day-differentiated cultures continuously treated with FBS or replenished with NPSC medium (Fig. 7L). Viral titers were undetectable in 16-day-differentiated cultures continuously treated with FBS until day 7 p.i. Also, 16-day-differentiated cultures replenished with NPSC medium supported very low levels of infectious virus compared to levels in infected NPSCs. These results suggest that the longer NPSCs are differentiated in the presence of FBS (5 days versus 16 days), the less these cells support CVB3 replication.

We analyzed viral protein expression levels and viral titers in NPSCs during the course of well-defined differentiation to determine the susceptibility of the three neural cell lineages to CVB3 infection (Fig. 8). The 5-day- and 16-day-differentiated cultures were infected with eGFP-CVB3 at low (MOI of 0.1)

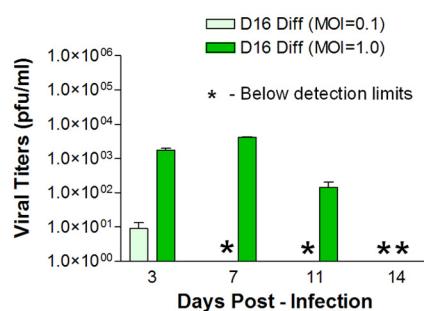
and high (MOI of 1.0) MOI. Differentiation medium was added back to infected cultures, and cells were harvested after a period of time (days 3 and 14 p.i.) based on the kinetics of viral protein expression, as determined for the experiment shown in Fig. 6. We evaluated infection by eGFP expression and the degree of NPSC differentiation utilizing nestin and three differentiation markers (GFAP, β -tubulin, and MBP) (Fig. 8A). For comparison, cells from 5-day-differentiated cultures were infected with eGFP-CVB3 at a low MOI (MOI of 0.1), cultured in the presence of NPSC medium, and stained for nestin or differentiation markers at day 2 p.i. As shown in Fig. 6, 5-day-differentiated cultures in NPSC medium showed relatively high levels of viral protein expression at day 2 p.i. Furthermore, these cultures expressed high levels of nestin as well as the three differentiation markers, suggesting that these



B NPSCs Differentiated For 5 Days + Diff Media



C NPSCs Differentiated For 16 Days + Diff Media



cells were not fully differentiated. The 5-day-differentiated cultures in differentiation medium showed less viral protein expression and more diffuse nestin staining. Higher-magnification images of hatched white boxes for 5-day-differentiated cultures in differentiation medium demonstrated colocalization of viral protein expression and either nestin-positive or β -tubulin-positive cells (Fig. 8A, white arrows). Also, we observed a lack of viral protein expression in 5-day-differentiated cultures in differentiation medium expressing high levels of GFAP or MBP (Fig. 8A, blue arrows). For the single time point examined before harvest (day 3 p.i.), low to moderate levels of viral titers were observed in cultures of 5-day-differentiated cells in differentiation medium (Fig. 8B), depending upon the initial viral inoculum. In contrast, 16-day-differentiated cultures in differentiation medium showed no viral protein expression at 14 days p.i., suggesting again that well-differentiated cells did not support CVB3 replication (as shown in Fig. 6). Furthermore, these cultures lacked nestin expression and expressed moderate to high levels of GFAP, β -tubulin, and MBP, which suggested their highly differentiated status. Also, low viral titers were observed until day 11 p.i. for cultures given a higher initial inoculum (MOI of 1.0) (Fig. 8C). These results suggest that the highly differentiated 16-day-differentiated cultures supported significantly less CVB3 replication than the 5-day-differentiated cultures.

Infection of NPSCs and alteration of the differentiation pathway. We inspected the ability of infected neurospheres to differentiate into the three downstream cell lineages following treatment with FBS. C57BL/6 NPSCs were mock infected or infected with eGFP-CVB3 (MOI of 0.1). In parallel, actin-GFP NPSCs were mock infected or infected with DsRed-CVB3 (MOI of 0.1). After 2 days p.i., the NPSC cultures were treated with FBS (differentiation medium) for an additional 3 days (Fig. 9). Infected NPSC cultures were observed for virus protein expression by fluorescence microscopy before (day 2 p.i.) and after (day 3 p.i.) FBS treatment. Intriguingly, infected NPSCs appeared less attached or flattened in the presence of FBS at day 3 p.i. than mock-infected NPSCs. By fluorescence microscopy, infected NPSCs were shown to express detectable levels of viral protein at day 2 and day 3 p.i. After 3 days of FBS treatment (day 5 p.i.), infected NPSC cultures were fixed and stained for three neural cell lineage markers (GFAP, β -tubulin, and Olig2). Olig2 expression has been utilized previously in other studies to identify cells in the oligodendrocyte lineage (5). The amount of fluorescent signal for each marker was

quantified by ImageJ analysis, and the relative fluorescence was calculated as a percentage of the total number of cells for each stain. For C57BL/6 NPSCs, DAPI was utilized to calculate relative fluorescence values. For actin-GFP NPSCs, GFP signal was utilized to calculate relative fluorescence values.

We compared the relative fluorescence value for each marker between mock-infected and infected NPSCs differentiated with FBS. For C57BL/6 NPSCs, no statistically significant changes were observed in the relative fluorescence levels for all three markers following infection. In contrast, a statistically significant increase in relative β -tubulin levels ($P = 0.02$, Student's *t* test) was observed in actin-GFP NPSCs following infection, compared to mock-infected control cultures. Also, the percentage of GFAP⁺ cells was reduced in actin-GFP NPSCs following infection although not by a statistically significant level ($P = 0.06$, Student's *t* test). No statistically significant change was observed in the relative fluorescence levels of Olig2 within actin-GFP NPSCs following infection. A direct comparison of actin-GFP and C57BL/6 NPSCs was problematic due to the methodology applied in obtaining the ratios of the representative markers. For example, the downstream cell lineages for actin-GFP NPSCs were determined based on cytoplasmic GFP expression, compared to nuclear staining (DAPI) for C57BL/6 NPSCs. Alterations in the relative population of downstream cell lineages following differentiation of infected NPSCs may be dependent upon isolation differences during stem cell harvesting, isolation, and cell culture, as well as on potential stochastic differences inherent during infection and after the FBS treatment. Also, infected NPSCs were reduced in number at 5 days p.i. compared to the number of mock-infected NPSCs, suggesting either CPE or a reduction in cellular proliferation during the differentiation procedure. Each downstream progenitor cell lineage may be differentially susceptible to CVB3-induced CPE. These results indicate that CVB3 may bias neural stem cell differentiation by increasing the percentage of immature β -tubulin-positive neuroblasts.

DISCUSSION

The ability of CVB3 to target neural stem cells in the neonatal CNS raises many questions regarding stem cell function and normal brain development within the surviving host. We previously established a murine model for neonatal coxsackievirus B3 (CVB3) infection, and proliferating nestin-positive progenitor cells were identified as primary targets for early

FIG. 8. Reduction in CVB3 replication and viral protein expression in highly differentiated NPSCs expressing neural differentiation markers and lacking nestin expression. C57BL/6 NPSCs were differentiated in the presence of FBS for 5 or 16 days and infected with eGFP-CVB3 (MOI of 0.1 or 1.0). After infection, differentiated NPSCs were cultured in NPSC medium or differentiation medium. Also, viral titers were evaluated in these cultures up to 14 days p.i. (A) After day 2, 3, or 14 p.i., cultures were stained for nestin and three downstream cell lineage markers (GFAP, β -tubulin, and MBP). Moderate to high levels of all three cell lineage markers were observed in all cultures. Single-channel black and white images for insets show the expression level for each cell lineage marker. Also, little to no nestin expression was observed in cultures of 16-day-differentiated cells in differentiation medium, indicating well-defined differentiation of these cells. The highest level of viral protein expression was observed in 5-day-differentiated cells in NPSC medium. Detectable levels of viral protein expression were seen in 5-day-differentiated cells in differentiation medium. Higher magnification of boxed areas showed colocalization of viral protein expression in cells with moderate levels of diffuse nestin and in some β -tubulin-positive cells (white arrows). In contrast, a lack of colocalization was observed for GFAP and MBP staining (blue arrows). Little to no viral protein expression was observed in 16-day-differentiated cells in differentiation medium. (B) Cultures of 5-day-differentiated cells in differentiation medium supported low to moderate levels of viral replication depending upon the MOI utilized at the single time point analyzed. (C) In contrast, cultures of 16-day-differentiated cells in differentiation medium supported lower levels of viral replication at either MOI utilized, and these levels dropped to below detection limits at day 14 p.i.

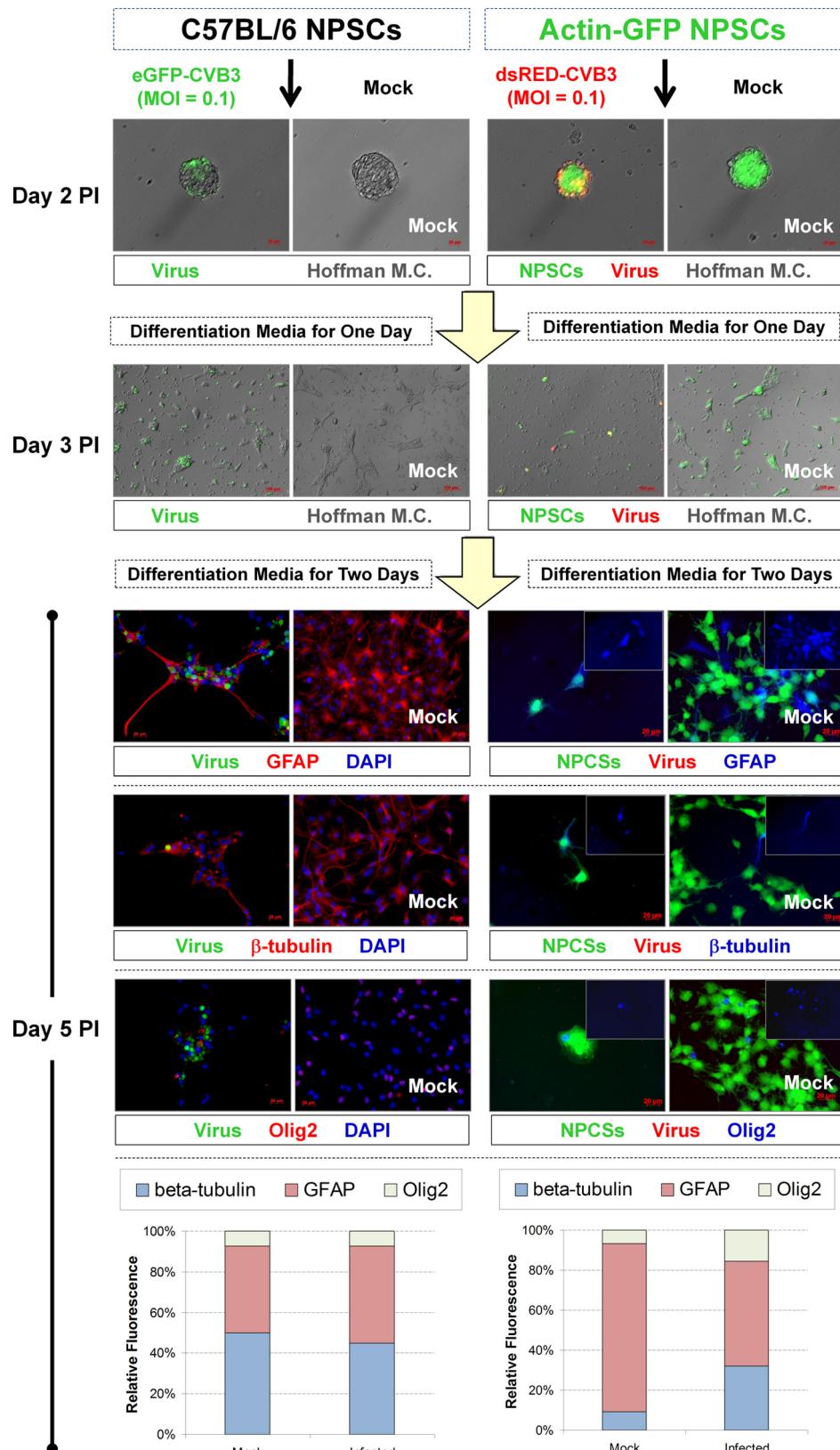


FIG. 9. CVB3 infection and alteration of the NPSC differentiation pathway. C57BL/6 NPSCs were infected with eGFP-CVB3 (MOI of 0.1). In parallel, actin-GFP NPSCs were infected with DsRed-CVB3 (MOI of 0.1). After day 2 p.i., infected NPSCs were differentiated in the presence of FBS for 3 days and harvested for immunofluorescence staining. Infected NPSCs were stained for three downstream cell lineage markers (GFAP, β-tubulin, and Olig2). For C57BL/6 NPSCs, the relative fluorescence of each marker following infection was calculated and normalized to DAPI signal using the ImageJ program. For actin-GFP NPSCs, the relative fluorescence of each marker following infection was calculated and normalized to GFP signal using the ImageJ software (inset images are shown for a single blue channel).

infection (15, 16). In addition, CVB3 established a persistent infection in mice surviving a neonatal infection, characterized by the continued detection of viral RNA by reverse transcription-PCR (RT-PCR), along with long-lasting lesions and chronic signs of inflammation and microgliosis up to 9 months following infection (17).

To more easily discriminate the effects of CVB3 infection on neural stem cell function and differentiation, we thought it imperative to duplicate any findings observed *in vivo*, in parallel with neural stem cells grown in culture. Given that CVB3 is a cytopathic virus, we wished to determine if neural stem cells were susceptible to infection and exhibited cytopathic effects (CPE) following infection. Therefore, we inspected the susceptibility of neurospheres or NPSCs grown in culture to CVB3 infection. Also, we wished to determine whether CVB3 infection might alter the differentiation path of NPSCs. The benefits of using NPSCs grown in culture include the ability of using a relatively pure population of target cells, controlling the multiplicities of infection (MOI) at the onset of infection, and discriminating the initial time of infection during time course assays, factors difficult to control *in vivo*.

Despite the benefits of examining infected NPSCs grown in culture, we realized that neurosphere aggregates form relatively complex mixtures of both stem and progenitor cells expressing a variety of cell markers. In fact, infection of cultured primary NPSCs may be a particularly intriguing and dynamic model of CVB3 infection, given the complex mixture of cell types with potentially differential antiviral responses induced during infection. In this regard, little is known about the ability of stem cells to induce and respond to interferon following infection. We expect that the interferon response within our primary stem cell culture system may be responsible for the observed carrier state infection observed in NPSCs, which may ultimately parallel the establishment of viral persistence within the CNS. Also, our results suggest that susceptibility to infection may reflect the heterogeneity of progenitor cells in neurosphere aggregates; each progenitor cell type may have a slightly different susceptibility to coxsackievirus infection and CPE depending upon the progenitor type or stage of differentiation. Also, some adherent cells with a flattened, stretched morphology remained in culture and appeared to be resistant to infection, as judged by viral protein expression. Adding to the complexity, susceptibility to infection may be altered as progenitor cells differentiate into the downstream lineages. A recent publication suggests preferential coxsackievirus replication within immature neurons expressing relatively high levels of coxsackievirus-adenovirus receptor (CAR) compared to their fully differentiated counterparts (1).

Two independent neurosphere isolations from C57BL/6 mice and actin promoter-GFP transgenic mice generated NPSCs which were shown to be highly susceptible to CVB3 infection. Also, virus-mediated cell death in NPSCs following CVB3 infection was observed by trypan blue staining. The ability of CVB3 to infect neonatal NPSCs and induce CPE may shed light on potential CNS development defects following neonatal infection. In addition, possible long-term consequences may be detected in the surviving host, given the substantial number of functional neural stem cells in the adult CNS. However, the capacity of adult NPSCs to support CVB3 infection remains to be determined. Also, distinct populations of NPSCs from dif-

ferent regions of the murine CNS may be differentially susceptible to infection. Our previous published *in vivo* data suggest that NPSCs in the subventricular zone (SVZ) and hippocampus are both susceptible to infection (15, 16). However, the degree of susceptibility and the ability to establish a persistent infection may be reflected by the particular anatomical location of isolated NPSCs.

Despite clear CPE in infected NPSCs following CVB3 infection, surviving neurospheres were observed in cultures replenished with complete NPSC medium. These results suggest that NPSCs may mount functional antiviral responses, perhaps by inducing and/or responding to interferon following infection (38). In this respect, very little is known regarding the ability of stem cells to induce innate immune responses in response to pathogens. It remains unknown if prolonged antiviral responses or chronic interferon may alter or compromise normal neural stem cell function and the proper generation of downstream cell lineages. The possibility remains that a particular nestin-positive stem cell population may exist within the neurosphere aggregate which is both resistant to CVB3 infection and yet can reestablish the NPSC culture. Nestin-positive progenitor cells have been previously shown to be responsible for neurosphere regeneration in culture (32).

Our results also indicate the ability of CVB3 to persist in NPSCs grown in culture. This finding may be particularly relevant, given our recent work indicating the ability CVB3 to persist in the adult CNS following neonatal infection (17). We speculate that neural stem cells in the adult CNS may be a site of viral persistence and that CVB3 may undergo active viral replication during stem cell proliferation and division. Conversely, we expect that viral replication and viral protein expression may become substantially reduced within quiescent primary stem cells, as opposed to rapidly proliferating progenitor cells (14). As we have shown, acute CVB3 replication may harm NPSCs in culture, and this injurious effect on stem cell function may occur *in vivo* as well. In fact, viral RNA by itself may be damaging to normal brain function, as shown for myocytes in culture (39). Also, the expression of viral proteins during CVB3 replication may activate the host immune response, which may compromise neural stem cell function.

We evaluated the ability of CVB3 to target neuronal, oligodendrocyte, and astrocyte precursor cells through immunofluorescence colocalization studies using antibodies against neuronal class III β -tubulin (immature neurons), NG2/Olig2 (oligodendrocyte precursor markers), myelin basic protein ([MBP] mature oligodendrocyte marker), and GFAP (glial/astrocyte precursor marker) (13, 22, 33). Oligodendrocytes are critical cells in the CNS and provide axons with insulating myelin sheaths. Astrocytes, once considered merely support cells in the brain, are now thought to play a more active role by affecting the activity of neurons (22). CVB3 appeared to preferentially target nestin-positive and NG2 $^{+}$ cells for infection. It is not clear from our studies which cells might preferentially undergo CPE following infection. However, any reduction in nestin-positive and NG2 $^{+}$ progenitor cells following infection/CPE might alter the percentage of downstream oligodendrocyte precursor cells and potentially impact normal myelination in the developing CNS. Also, well-differentiated cultures expressing high levels of GFAP, β -tubulin, or MBP and lacking nestin expression failed to support

CVB3 infection, as determined by viral protein expression or by viral titers. Of note, differentiation of infected actin-GFP NPSCs resulted in an increase of neuronal class III β -tubulin-expressing cells compared to levels in mock-infected controls. However, no statistically significant changes within infected C57BL/6 NPSCs were observed following their differentiation.

In summary, we propose that CVB3 may target neural stem cells in culture, induce CPE preferentially in progenitor cells compared to their differentiated counterparts, and may persist in neurosphere cultures replenished with fresh complete NPSC medium. Taken together, our results suggest that virus protein expression was robust in undifferentiated neurospheres, yet differentiated cells adjacent to infected neurospheres appeared to be refractory to infection. Future studies will evaluate neural stem cell function and potential genomic alterations in the CVB3 genome within persistently infected NPSC cultures. Also, actin-GFP NPSCs will assist us with future experiments designed to track previously infected neural stem cells adaptively transferred within a new host. We will test the ability of NPSCs surviving infection to continue functioning normally, give rise to the three downstream neural cell lineages, and migrate correctly within an *in vivo* environment.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health awards R01 NS054108 (to R.F.), R01 AI042314, and HL093177 (to J.L.W.), a National Institutes of Mental Health Minority Research Infrastructure Support Program R24 Faculty Fellow Award MH065515 (to R.F.), an SDSU University Grants Program Award (to R.F.), and two Achievement Rewards for College Scientists Foundation Scholarships (to G.T. and J.M.T.-G.).

We declare that we have no conflicts of interest.

REFERENCES

- Ahn, J., et al. 2008. Primary neurons become less susceptible to coxsackievirus B5 following maturation: the correlation with the decreased level of CAR expression on cell surface. *J. Med. Virol.* **80**:434–440.
- Alexanian, A. R., and S. N. Kurpad. 2005. Quiescent neural cells regain multipotent stem cell characteristics influenced by adult neural stem cells in co-culture. *Exp. Neurol.* **191**:193–197.
- Alvarez-Buylla, A., B. Seri, and F. Doetsch. 2002. Identification of neural stem cells in the adult vertebrate brain. *Brain Res. Bull.* **57**:751–758.
- Baracskay, K. L., G. J. Kidd, R. H. Miller, and B. D. Trapp. 2007. NG2-positive cells generate A2B5-positive oligodendrocyte precursor cells. *Glia* **55**:1001–1010.
- Chandran, S., et al. 2003. FGF-dependent generation of oligodendrocytes by a hedgehog-independent pathway. *Development* **130**:6599–6609.
- Chapman, N. M., and K. S. Kim. 2008. Persistent coxsackievirus infection: enterovirus persistence in chronic myocarditis and dilated cardiomyopathy. *Curr. Top. Microbiol. Immunol.* **323**:275–292.
- Cheeran, M. C., et al. 2005. Neural precursor cell susceptibility to human cytomegalovirus diverges along glial or neuronal differentiation pathways. *J. Neurosci. Res.* **82**:839–850.
- Crocker, S. J., R. F. Frausto, J. L. Whitton, and R. Milner. 2008. A novel method to establish microglia-free astrocyte cultures: comparison of matrix metalloprotease expression profiles in pure cultures of astrocytes and microglia. *Glia* **56**:1187–1198.
- Das, S., and A. Basu. 2008. Japanese encephalitis virus infects neural progenitor cells and decreases their proliferation. *J. Neurochem.* **106**:1624–1636.
- Das, S., S. Chakraborty, and A. Basu. 2010. Critical role of lipid rafts in virus entry and activation of phosphoinositide 3' kinase/Akt signaling during early stages of Japanese encephalitis virus infection in neural stem/progenitor cells. *J. Neurochem.* **115**:537–549.
- Das, S., D. Ghosh, and A. Basu. 2009. Japanese encephalitis virus induces immuno-competency in neural stem/progenitor cells. *PLoS One* **4**:e8134.
- David, P., et al. 1993. MRI of acute disseminated encephalomyelitis after coxsackie B infection. *J. Neuroradiol.* **20**:258–265.
- Dawson, M. R., A. Polito, J. M. Levine, and R. Reynolds. 2003. NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. *Mol. Cell Neurosci.* **24**:476–488.
- Feuer, R., I. Mena, R. Pagarigan, M. K. Slifka, and J. L. Whitton. 2002. Cell cycle status affects coxsackievirus replication, persistence, and reactivation in vitro. *J. Virol.* **76**:4430–4440.
- Feuer, R., et al. 2003. Coxsackievirus B3 and the neonatal CNS: the roles of stem cells, developing neurons, and apoptosis in infection, viral dissemination, and disease. *Am. J. Pathol.* **163**:1379–1393.
- Feuer, R., et al. 2005. Coxsackievirus targets proliferating neuronal progenitor cells in the neonatal CNS. *J. Neurosci.* **25**:2434–2444.
- Frisk, G., M. A. Lindberg, and H. Diderholm. 1999. Persistence of coxsackievirus B4 infection in rhabdomyosarcoma cells for 30 months. *Brief report. Arch. Virol.* **144**:2239–2245.
- Gage, F. H. 2000. Mammalian neural stem cells. *Science* **287**:1433–1438.
- Graber, D., C. Fossoud, E. Grouteau, C. Gayet-Mengelle, and J. P. Carriere. 1994. Acute transverse myelitis and coxsackie A9 virus infection. *Pediatr. Infect. Dis. J.* **13**:77.
- Hack, M. A., M. Sugimori, C. Lundberg, M. Nakafuku, and M. Gotz. 2004. Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol. Cell Neurosci.* **25**:664–678.
- Horner, P. J., and T. D. Palmer. 2003. New roles for astrocytes: the nightlife of an “astrocyte.” *La vida loca! Trends Neurosci.* **26**:597–603.
- Kim, K. S., N. M. Chapman, and S. Tracy. 2008. Replication of coxsackievirus B3 in primary cell cultures generates novel viral genome deletions. *J. Virol.* **82**:2033–2037.
- Kim, K. S., et al. 2005. 5'-Terminal deletions occur in coxsackievirus B3 during replication in murine hearts and cardiac myocyte cultures and correlate with encapsidation of negative-strand viral RNA. *J. Virol.* **79**:7024–7041.
- Klingel, K., et al. 1992. Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: quantitative analysis of virus replication, tissue damage, and inflammation. *Proc. Natl. Acad. Sci. U. S. A.* **89**:314–318.
- Luo, M. H., et al. 2010. Human cytomegalovirus infection causes premature and abnormal differentiation of human neural progenitor cells. *J. Virol.* **84**:3528–3541.
- Luo, M. H., P. H. Schwartz, and E. A. Fortunato. 2008. Neonatal neural progenitor cells and their neuronal and glial cell derivatives are fully permissive for human cytomegalovirus infection. *J. Virol.* **82**:9994–10007.
- Mokry, J., J. Karbanova, and S. Filip. 2005. Differentiation potential of murine neural stem cells in vitro and after transplantation. *Transplant. Proc.* **37**:268–272.
- Odeberg, J., et al. 2006. Human cytomegalovirus inhibits neuronal differentiation and induces apoptosis in human neural precursor cells. *J. Virol.* **80**:8929–8939.
- Odeberg, J., et al. 2007. Late human cytomegalovirus (HCMV) proteins inhibit differentiation of human neural precursor cells into astrocytes. *J. Neurosci. Res.* **85**:583–593.
- Okamoto, S., et al. 2007. HIV/gp120 decreases adult neural progenitor cell proliferation via checkpoint kinase-mediated cell-cycle withdrawal and G₁ arrest. *Cell Stem Cell* **1**:230–236.
- Park, J. H., et al. 2007. Genetic modification does not affect the stemness of neural stem cells in nestin promoter-GFP transgenic mice. *Neurosci. Lett.* **421**:185–190.
- Rogister, B., T. Ben-Hur, and M. Dubois-Dalcq. 1999. From neural stem cells to myelinating oligodendrocytes. *Mol. Cell Neurosci.* **14**:287–300.
- Rothenaigner, I., et al. 2007. Long-term HIV-1 infection of neural progenitor populations. *AIDS* **21**:2271–2281.
- Sawyer, M. H. 2002. Enterovirus infections: diagnosis and treatment. *Semin. Pediatr. Infect. Dis.* **13**:40–47.
- Sharma, N., et al. 2009. Functional role of the 5' terminal cloverleaf in coxsackievirus RNA replication. *Virology* **393**:238–249.
- Tabor-Godwin, J. M., et al. 2010. A novel population of myeloid cells responding to coxsackievirus infection assists in the dissemination of virus within the neonatal CNS. *J. Neurosci.* **30**:8676–8691.
- Wellen, J., J. Walter, P. Jangouk, H. P. Hartung, and M. Dihne. 2009. Neural precursor cells as a novel target for interferon-beta. *Neuropharmacology* **56**:386–398.
- Wessely, R., A. Henke, R. Zell, R. Kandolf, and K. U. Knowlton. 1998. Low-level expression of a mutant coxsackieviral cDNA induces a myopathic effect in culture: an approach to the study of enteroviral persistence in cardiac myocytes. *Circulation* **98**:450–457.