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Original Article

Inhibitory effects of murine cytomegalovirus infection on neural stem/progenitor cells of embryonic mouse brains

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Abstract: Introduction: The aim of this study was to examine the effects of murine cytomegalovirus (MCMV) infection on neural stem/progenitor cells (NSPCs) *in vitro*. Materials and Methods: NSPCs infected by MCMV at a multiplicity of infections (MOIs) of 5, 1, or 0.1 were cultured in a differentiation medium. Ratio of differentiated cells was detected by flow cytometry. Protein markers for NSPCs and differentiated cell lineages were investigated with immunofluorescent staining. MCMV infection was traced by MCMV early antigen. MicroRNA expression of proteins related to Wnt signal pathways was measured with real-time RT-PCR. Results: Non-infected control NSPCs proliferated to form neurospheres and differentiated into neurofilament 200-positive neurons or glial fibrillary acidic protein-positive astrocytes. However, MCMV-infected NSPCs differentiated more slowly with less nestin. Moreover, the ratio of astrocytes, neurons, and mRNA levels of Wnt-3, Wnt-7a, Ngn-2, c-Myc, and cyclin D1 in infected NSPCs was significantly lower than the control group. Early antigens were consistently detected in infected NSPCs. These changes positively correlated with multiplicity of infections for the virus infection. Discussion: MCMV infection significantly inhibits differentiation of NSPCs and inactivates Wnt signal pathways during cell differentiation. This inhibitory effect may be a primary cause of brain development abnormalities in congenital cytomegalovirus infection.

Keywords: Cytomegalovirus, neural stem/progenitor cells, differentiation, Wnt signal pathways

Introduction

Congenital cytomegalovirus (CMV) infection is an important cause of abnormal brain development in fetuses. Exploring the mechanisms of encephalodysplasia induced by CMV may further understanding of these abnormalities and help establish an effective strategy to prevent this congenital malformation of the central nervous system [1-3].

In infants, lesions that result from CMV infection are mainly located in the periventricular region, with inflammation and calcification [4]. However, little is known about the pathogenesis of lesions at the molecular level. The pathology of congenital murine CMV (MCMV) infection in the central nervous system is similar to congenital human CMV infection. Preliminary data for this study indicated that central nervous system lesions resulting from MCMV infection are located in the periventricular region, as previously reported [4]. It has been well established in the literature that the periventricular region is enriched with neural stem/progenitor cells (NSPCs) during embryonic development [5]. This preliminary finding also revealed that CMV inhibits NSPC proliferation by producing virions after infection (data not published).

NSPCs proliferate and differentiate into different nerve cells which constitute brain tissues. However, no efforts have been made to examine the effects of CMV infection on NSPC differentiation. During development of normal mouse embryos, NSPCs express nestin protein, a special marker of NSPCs. Neuronal precursors and neuroglial precursors, that differenti-

ate from NSPCs, decrease expression of nestin protein while increasing expression of NSE protein or GFAP. Eventually, these precursor cells mature into neurons or astrocytes, respectively. NSPCs develop into mature neurons and astrocytes in approximately three weeks [6-8].

This present study aimed to investigate the effects of MCMV infection on differentiation of NSPCs in an *in vitro* cell culture model. Possible underlying mechanisms responsible for these effects were also explored.

Materials and methods

Virus

To study the effects of virus infection on NSPCs, the Smith strain of MCMV was obtained from Hubei Province Academy of Preventive Medicine and Sciences. Mouse embryo fibroblast culture-adapted MCMV was then stored at 4°C. The virus titer was evaluated by a plaque-forming assay.

Mice

All animal protocols were approved by the Medical Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and conformed to Public Health Service Guide for the Care and Use of Laboratory Animals.

BALB/C mice (8-10 weeks old) were provided by Hubei Province Academy of Preventive Medicine and Sciences. Mice, with a female-to-male ratio of 2:1, were kept in the Medical Animal Center of Tongji Hospital. Pregnancies were dated as beginning on embryonic day 0 (E0) on the morning of the day a vaginal seminal plug was found after overnight mating.

Reagents

Reagents used included: fetal calf serum (Hyclone, Logan, UT), high-glucose Dulbecco's modified Eagle's medium (DMEM-H; Hyclone, Logan, UT), DMEM/Ham's F-12 nutrient mixture (DMEM/F12) medium (Hyclone, Logan, UT), mouse epidermal growth factor (mEGF) and basic fibroblast growth factor (bFGF) (both from Pepro Tech, Rocky Hill, NJ), and B27 (Gibco, Grand Island, NY). The following antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA): Nestin, GFAP, Neurofilament 200 kDa (NF200), NSE, and their secondary antibodies.

Cell culture

Mouse embryonic brain tissues were isolated from the BALB/c mice at 13.5 days gestation, as described previously [9]. Briefly, the meninges were gently dissected free and brains were washed with phosphate-buffered saline (PBS) to remove any remaining blood. The brains were triturated, repeatedly, to yield a mixture of single cells. Cell suspension was filtered with a 200-mesh sieve, centrifuged at 200 × *g* for 5 minutes, and then cultured at a density of 1×10^5/mL in DMEM/F12 supplemented with 2% B27, 20 ng/mL EGF, and bFGF in an atmosphere of saturated humidity at 37°C and 5% CO_2. Medium was replaced once every 3 days and passage of cells was performed every 6 to 7 days. Cells passaged 3-5 times were used in the experiment.

Identification of NSPCs

Sub-cultured cells were fixed with 4% paraformaldehyde and transferred to a coverslip coated with polylysine. Cells were permeabilized with 0.1% Triton X-100 and blocked with normal non-immune animal serum. They were incubated with primary antibody goat anti mouse nestin (1:200) for 1 hour at 37°C and then with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (1:200) for 45 minutes at 37°C. Coverslips were sealed with 30% glycerol in PBS and then observed under a confocal microscope (Olympus FV 500).

Differentiation potential of NSPCs

NSPCs were inoculated in 6-well plates with coverslips, previously coated with polylysine, and cultured in DMEM/F12 differentiation medium with 2% fetal calf serum for 5-7 days. Cells were permeabilized with 0.1% Triton X-100, blocked with normal non-immune animal serum, and co-incubated with rabbit anti-mouse GFAP (1:200) and mouse anti-mouse NSE (1:200) for 1 hour at 37°C as well as with FITC-labeled anti-mouse IgG (1:200) for 45 minutes at 37°C. Coverslips were sealed with

30% glycerol in PBS and observed under a confocal microscope (Olympus FV 500).

Expression of GFAP and NSE proteins was measured with immunofluorescence staining. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes. Coverslips were sealed with 30% glycerol in PBS and observed under a confocal microscope (Olympus FV 500).

Observation of morphological differentiation of NSPCs infected by MCMV

NSPCs were infected by MCMV with a multiplicity of infection (MOI) equal to one, incubated for 1 hour at 37°C, and centrifuged. Supernatant was discarded and cells were cultured in 6-well plates in DMEM/F12 differentiation medium with 2% fetal calf serum and with polylysine-coated coverslips. Cells were collected on days 3, 6, 9, 12, 15, 18, and 21. Expression of nestin, GFAP, and NSE proteins was detected by immunofluorescence staining. Fluorescence intensity was assessed using an HMIAS-2000 color pathologic analysis system. Ten visual fields were randomly selected for data acquisition. Expression of early antigens of MCMV (1:1500) was detected to confirm infection.

Detection of the differentiation ratio of infected NSPCs by flow cytometry

MCMV-infected NSPCs at MOIs of 5, 1, or 0.1 were cultured in a differentiation medium. Cells were digested with 0.25% trypsin at 3, 6, and 9 days after incubation in a differentiation culture, fixed with 4% paraformaldehyde, and permeabilized with 0.01% Triton X-100. Cells were serially incubated with primary antibodies nestin (1:200), GFAP (1:200), and NSE (1:200) for 1 hour at 37°C, then with FITC-labeled anti-mouse IgG (1:100) for 45 minutes at 37°C, and finally were detected via flow cytometry (BD, LSRII).

Detection of expression of differentiation genes of infected NSPCs by SYBR green-based real-time RT-PCR

MCMV-infected NSPCs at MOIs of 5, 1, or 0.1 were cultured in a differentiation medium. Cells were collected after 12 hours and 1, 2, 3, 4, and 5 days after incubation in the differentiation culture. Total RNA of cells was extracted with TRIzol Reagent. Purity measured with ultraviolet spectrophotometry was in the range of 1.72 to 1.85. Two micrograms of RNA from every specimen served as the template for producing the first strand of cDNAs. Target genes and GAPDH genes from each sample were simultaneously analyzed.

Primer sequences were: Wnt-3 forward 5'-ATC CAG GAG TGC CAG CAT CAG-3' and reverse 5'-ATG CAC GAA GGC CGA TTC A-3'; Wnt-7a forward 5'-CGG ACG CTC ATG AAC TTA CAC AA-3' and reverse 5'-GGT ACA GGA GCC TGA CAC ACC A-3'; Ngn-2 forward 5'-CAT TTG CAA TGG CTG GCA TC-3' and reverse 5'-CAA TAG GCA TTG TGA CGA ATC TGG-3'; C-myc forward 5'-GCT CGC CCA AAT CCT GTA CCT-3' and reverse 5'-TCT CCA CAG ACA CCA CAT CAA TTT C-3'; Cyclin D1 forward 5'-AAT CGT GGC CAC CTG GAT G-3' and reverse 5'-CTT CAA GGG CTC CAG GGA CA-3'; GAPDH forward 5'-GCA CAG TCA AGG CCG AGA A-3' and reverse 5'-CCT CAC CCC ATT TGA TGT TAG TG-3'.

Total volume of the amplification reaction solution was 25 μL, consisting of 12.5 μL SYBR Premix Ex Taq, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL Rox reference dye II (Takara), and 1 μL sample cDNA. Initial denaturation consisted of 1 cycle at 95°C for 10 seconds, 45 cycles at 95°C for 5 seconds, and 58°C for 20 seconds.

Since the standard curve of PCR reaction of target genes was similar in amplification efficiency to GAPDH genes, data were analyzed using the $2^{-\Delta\Delta}$ CT method, with CT as the cycle threshold, as described previously [10]. ΔCT and ΔΔCT values of each sample, at each experimental timepoint, were calculated as: $\Delta CT = (CT_{target} - CT_{GAPDH})$, $\Delta\Delta CT = (\Delta CT_{target} - \Delta CT_{calibrator})$.

$\Delta CT_{calibrator}$ is the ΔCT value of the normal control group at 12 hours. The value of $2^{-\Delta\Delta}$ CT represents relative fold changes between the calibrator and target gene.

Data processing and statistical analyses

All data collected from the experiment were analyzed using SPSS software and are presented as mean ± standard deviation. Student's *t*-test was used for evaluation of differences between groups, with $P < 0.05$ indicating statistical significance.

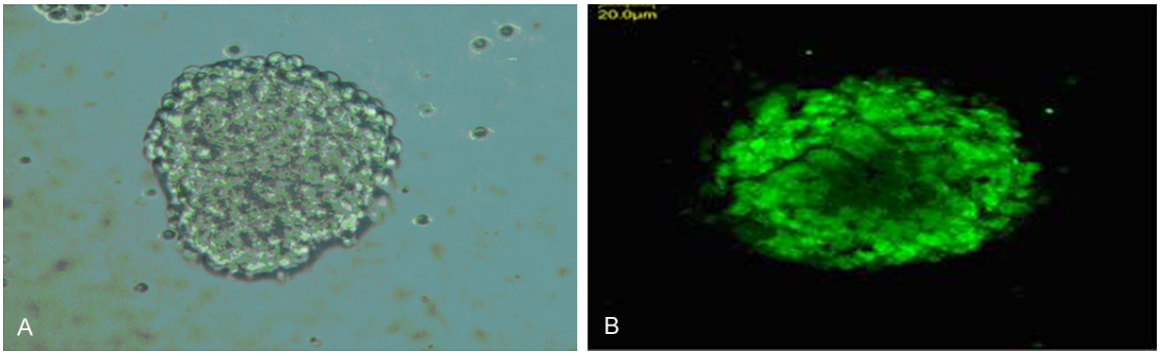


Figure 1. Formation of neurospheres and identification of NSPCs. A. A neurosphere in the control group (400 ×). B. NSPCs in the control group expressed nestin.

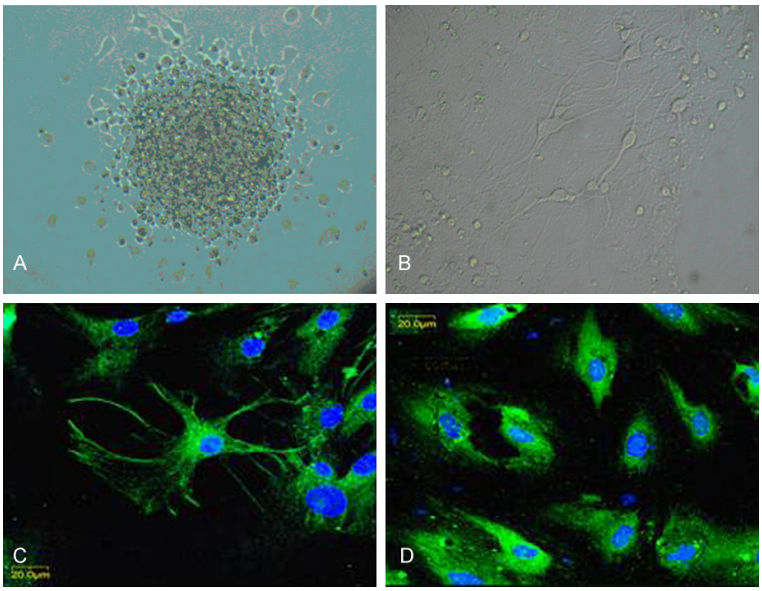


Figure 2. Differentiation assay. A. Differentiation of NSPCs at day 1 in the control group (400 ×). B. Differentiation of NSPCs at day 7 (400 ×). C. Astrocytes differentiated from NSPCs expressed GFAP. D. Neurons differentiated from NSPCs expressed NF-200.

Results

Culture and identification of NSPCs

Primary cultured cells consisted of single cells in suspension, gradually forming multicellular neurospheres after 5 or 6 days. These clones were round and grew in suspension with typical cellular morphology without obvious neurite outgrowth. Cells formed neurospheres after 3-5 passages and expressed nestin protein, indicating that they were NSPCs (**Figure 1A**, **1B**).

Differentiation potential of NSPCs

NSPCs gradually adhered to the culture dish wall, 12 hours after differentiation. Cells at the edge of the neurospheres became spindle-shaped and grew into neurites. Neurites appeared radially around the neurospheres (**Figure 2A**). Neurospheres gradually lost their typical shape, 2 or 3 days after differentiation, but gained an appearance of migrated and differentiated cells with intermingled processes within 5 to 7 days, similar to those of neurons and astrocytes (**Figure 2B**). Immunofluorescence staining results showed GFAP-positive astrocytes and NF-200-positive neurons (**Figure 2C**, **2D**).

Morphological observation on differentiation of NSPCs infected by MCMV

NSPCs in the infected groups did not show obvious morphological changes on the first day of differentiation. They seemed unable to adhere to the culture dish wall firmly. NSPCs infected at MOI = 5 or MOI = 1 appeared swollen, at 2 to 3 days, and detached from the edge of the neurospheres. The structure of neurospheres became loose and lost its spherical shape. Cell morphological changes in the group infected with MOI = 0.1 were slightly slower than those in the other two groups infected with higher MOIs. At 6 and 9 days, more swollen exfoliated cells were observed, with some cells containing an inclusion body and cells in the neurospheres separated from each other (**Figure 3A**). Cell debris increased while volume of the neuro-

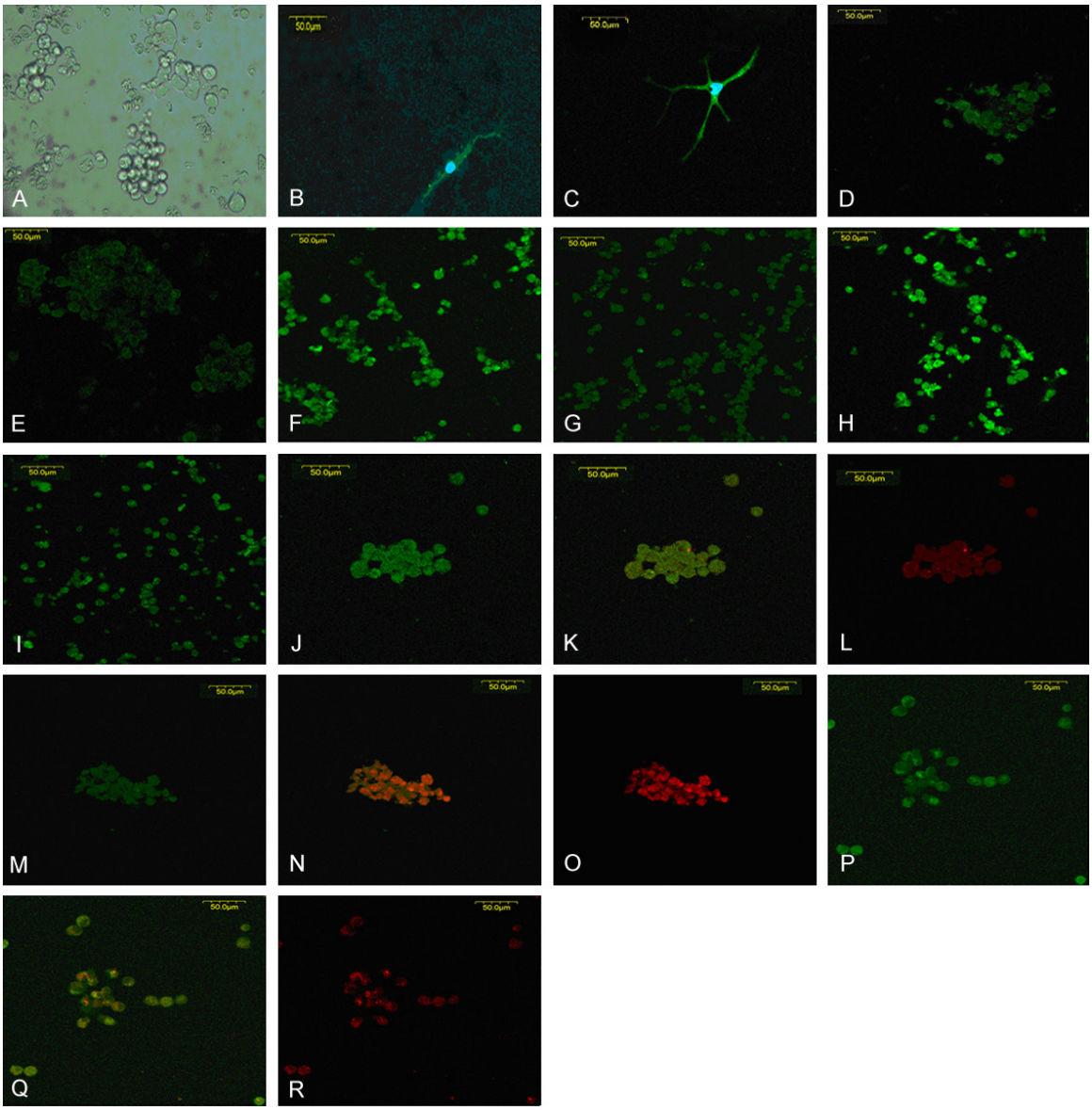


Figure 3. Neuronal differentiation of control group and expression of Nestin, GFAP, and NSE in the infected groups. (A) NSPCs were infected by MCMV for 5 d (400 ×). (B) Bipolar neurons differentiated from NSPCs expressed NSE at 12 d. (C) Multipolar neurons differentiated from NSPCs expressed NSE at 21 d. (D-I) Expression of GFAP and NSE in the infected groups at days 3, 12, and 21. (J-L) expression of Nestin (green), MCMV early antigen (red) and merged image (K) in the infected groups at day 3. (M-O) Expression of GFAP (green), MCMV early antigen (red) and a merged image (N) in the infected groups at day 12. (P-R) Expression of NSE (green), MCMV early antigen (red), and a merged image (Q) in the infected groups at day 21.

spheres decreased continuously and simultaneously. These results show that cell morphology change was associated with MOI dosage.

As observed in immunofluorescence studies, NSPCs in the infected groups had no neurites but gradually dispersed and scattered into single cells. Their morphology was significantly different from that of the control group (**Figure 3B-I**). Although cells showed positive staining for GFAP and NSE, markers of differentiated cells, MCMV early antigens (**Figure 3J-R**), and nestin in infected groups decreased very slowly and were significantly higher than the control group at each timepoint. However, GFAP and NSE levels in infected groups were significantly lower than those in the control group ($P < 0.05$; **Table 1**, **Figure 4**).

Table 1. Ratio of positive cells expressing nestin, GFAP, and NSE

		Control	MCMV infection, MOI		
			0.1	1	5
Nestin	3 d	40.50 ± 0.75	58.74 ± 1.06[a]	70.90 ± 0.69[a,b]	83.20 ± 1.04[a,b,c]
	6 d	37.77 ± 0.47	43.13 ± 1.34[a]	60.73 ± 0.64[a,b]	74.67 ± 0.86[a,b,c]
	9 d	31.60 ± 0.82	38.50 ± 1.35[a]	56.57 ± 0.80[a,b]	70.20 ± 0.79[a,b,c]
GFAP	3 d	58.43 ± 0.96	50.07 ± 1.53[a]	37.47 ± 0.87[a,b]	27.13 ± 0.91[a,b,c]
	6 d	64.50 ± 0.82	47.30 ± 0.95[a]	35.43 ± 1.26[a,b]	24.47 ± 0.86[a,b,c]
	9 d	69.60 ± 0.71	42.63 ± 1.53[a]	30.53 ± 0.67[a,b]	22.57 ± 0.86[a,b,c]
NSE	3 d	27.87 ± 0.31	18.63 ± 1.01[a]	16.03 ± 0.47[a]	8.27 ± 0.67[a,b,c]
	6 d	34.37 ± 1.15	17.00 ± 0.80[a]	14.07 ± 0.74[a,b]	9.23 ± 0.57[a,b,c]
	9 d	32.56 ± 0.76	13.40 ± 0.30[a]	12.83 ± 0.31[a]	8.77 ± 0.60[a,b,c]

[a]Compared with the control group, $P < 0.05$; [b]Compared with MOI = 0.1 group after MCMV infection, $P < 0.05$; [c]Compared with MOI = 1 group after MCMV infection, $P < 0.05$.

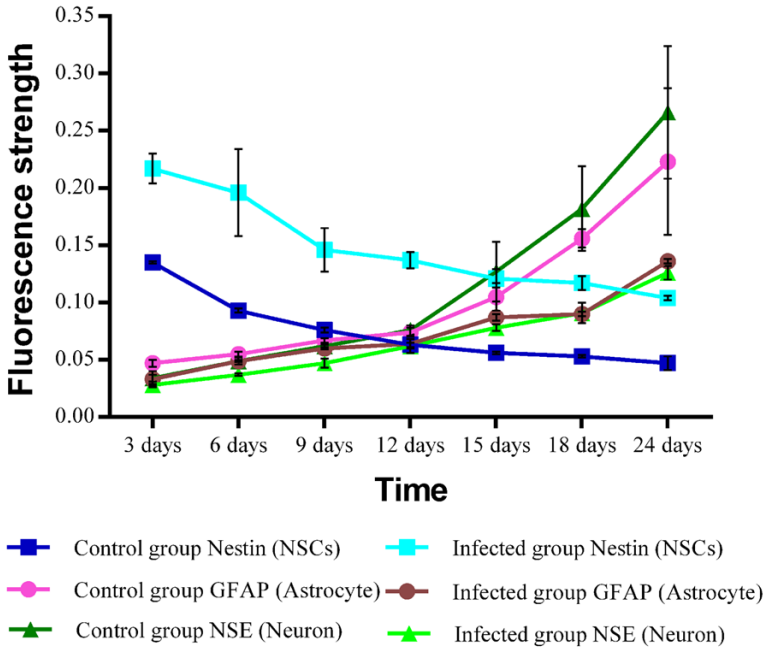


Figure 4. Comparison of the fluorescence strength of nestin, GFAP, and NSE.

Infected NSPCs were less able to differentiate into neurons and astrocytes

The proportion of nestin-positive cells in the control group steadily decreased and ratio of GFAP- and NSE-positive cells steadily increased after the start of differentiation. However, in infected groups, although change in ratio of nestin-positive cells showed a downward trend, the ratio was significantly higher than the control group ($P < 0.05$). Ratio of GFAP- and NSE-positive cells in infected groups was significantly lower than the control group ($P < 0.05$). Differences between the infected groups and control group became more obvious as the dose of MOIs increased. This result suggests that MCMV could inhibit differentiation of NSPCs into NSE-positive neurons and GFAP-positive astrocytes and this effect was dose-dependent with MCMV MOI (**Table 1**).

Expression of differentiation genes of infected NSPCs

Levels of Wnt-3 mRNA in the control group decreased slowly after 12 hours of incubation in the differentiation culture. Wnt-3 mRNA in the infected groups decreased from 12 hours to 2 days, then increasing slightly. MicroRNA levels in infected groups with MOI = 5 were 1.67- to 80-fold lower than the control group at each timepoint examined ($P < 0.05$; **Table 2**). Levels in infected group with MOI = 1 were significantly lower than the control group from 12 hours to 3 d, and in the MOI = 0.1 from day 1 to 2 (both, $P < 0.05$). Levels in the MOI = 5 group were lower than that of the MOI = 1 group from 12 hours to 1 day and in MOI = 0.1 group from day 3 to 5 ($P < 0.05$; **Table 2**). Amplification curves of GAPDH and Wnt-3 are shown in **Figure 5A**, **5B**.

Levels of Wnt-7a mRNA in the control group increased rapidly after 12 hours, reaching a peak at day 1. The mRNA levels remained steady from day 4 to 5 of differentiation culture (**Figure 5C**). Levels of Wnt-7a mRNA in all infected groups were lower than those in control group from 12 hours to 2 days ($P < 0.05$; **Table 2**), then increased slightly, reaching a peak at day 3. Wnt-7a mRNA levels in MOI = 5 group were significantly lower than the control group at day 3. Moreover, at all timepoints examined, Wnt-7a mRNA levels of the MOI = 5 group were

Table 2. Levelsof differentiation-related mRNAs in NSPCs

mRNA from NSPCs	Time	Control	MCMV infection, MOI		
			0.1	1	5
Wnt-3	12 h	1.00 ± 0.05	0.95 ± 0.16	0.89 ± 0.04[a]	0.60 ± 0.03[a,b,c]
	1 d	0.87 ± 0.08	0.10 ± 0.01[a]	0.09 ± 0.01[a]	0.01 ± 0.00[a,b,c]
	2 d	0.71 ± 0.06	0.01 ± 0.01[a]	0.04 ± 0.00[a]	0.01 ± 0.00[a]
	3 d	0.39 ± 0.07	0.32 ± 0.09	0.19 ± 0.07[a]	0.09 ± 0.01[a,b,c]
	4 d	0.38 ± 0.05	0.42 ± 0.01	0.37 ± 0.04	0.17 ± 0.01[a,b,c]
	5 d	0.31 ± 0.03	0.30 ± 0.02	0.33 ± 0.02	0.06 ± 0.01[a,b,c]
Wnt-7a	12 h	1.00 ± 0.05	0.88 ± 0.02[a]	0.87 ± 0.04 a	0.69 ± 0.04[a,b,c]
	1 d	5.78 ± 0.51	0.76 ± 0.02[a]	0.61 ± 0.03[a,b]	0.30 ± 0.03[a,b,c]
	2 d	3.77 ± 0.33	0.12 ± 0.01[a]	0.04 ± 0.00[a,b]	0.02 ± 0.00[a,b,c]
	3 d	1.97 ± 0.36	2.38 ± 0.13	2.09 ± 0.08[b]	1.30 ± 0.12[a,b,c]
	4 d	0.91 ± 0.33	2.13 ± 0.19[a]	1.36 ± 0.13[b]	0.68 ± 0.02[b,c]
	5 d	0.94 ± 0.09	2.30 ± 0.12[a]	1.85 ± 0.22[a,b]	0.90 ± 0.06[b,c]
Ngn-2	12 h	1.00 ± 0.21	1.07 ± 0.11	0.91 ± 0.02	1.12 ± 0.17
	1 d	2.84 ± 0.30	0.57 ± 0.02[a]	0.63 ± 0.04[a]	0.57 ± 0.06[a]
	2 d	0.21 ± 0.09	0.41 ± 0.01[a]	0.39 ± 0.01[a]	0.19 ± 0.06[b,c]
	3 d	0.14 ± 0.06	0.48 ± 0.05[a]	0.39 ± 0.07[a]	0.30 ± 0.02[a,b]
	4 d	0.10 ± 0.04	1.26 ± 0.05[a]	0.98 ± 0.09[a,b]	0.75 ± 0.03[a,b,c]
	5 d	0.04 ± 0.01	1.93 ± 0.17[a]	1.71 ± 0.19[a]	1.17 ± 0.11[a,b,c]
c-Myc	12 h	1.00 ± 0.02	0.94 ± 0.13	0.59 ± 0.05[a,b]	0.65 ± 0.09[a,b]
	1 d	2.12 ± 0.22	0.06 ± 0.02[a]	0.06 ± 0.00[a]	0.07 ± 0.01[a]
	2 d	2.54 ± 0.30	0.85 ± 0.08[a]	0.71 ± 0.01[a]	0.66 ± 0.13[a]
	3 d	1.79 ± 0.26	0.93 ± 0.05[a]	0.64 ± 0.05[a,b]	0.66 ± 0.09[a,b]
	4 d	1.54 ± 0.20	0.83 ± 0.11[a]	0.48 ± 0.09[a,b]	0.39 ± 0.08[a,b]
	5 d	2.41 ± 0.24	2.12 ± 0.17	1.79 ± 0.12[a]	1.54 ± 0.18[a,b]
Cyclin D1	12 h	1.00 ± 0.05	0.06 ± 0.01[a]	0.07 ± 0.02[a]	0.12 ± 0.02[a,b,c]
	1 d	0.56 ± 0.09	0.16 ± 0.02[a]	0.05 ± 0.01[a,b]	0.02 ± 0.01[a,b,c]
	2 d	1.20 ± 0.21	1.36 ± 0.17	1.39 ± 0.10	1.73 ± 0.16[a,b,c]
	3 d	0.64 ± 0.12	1.03 ± 0.07[a]	1.14 ± 0.10[a]	1.22 ± 0.16[a]
	4 d	0.81 ± 0.11	1.03 ± 0.14	0.95 ± 0.08	0.67 ± 0.04[b,c]
	5 d	1.02 ± 0.15	1.13 ± 0.24	0.97 ± 0.07	0.73 ± 0.10[a,b,c]

[a]Compared with the control group, $P < 0.05$; [b]compared with MOI = 0.1 group after MCMV infection, $P < 0.05$; [c]compared with MOI = 1 group after MCMV infection, $P < 0.05$.

lower than MOI = 1 and MOI = 0.1 groups ($P < 0.05$; **Table 2**). Furthermore, mRNA levels in the MOI = 1 group were lower than in MOI = 0.1 group from day 1 to 5 ($P < 0.05$; **Table 2**).

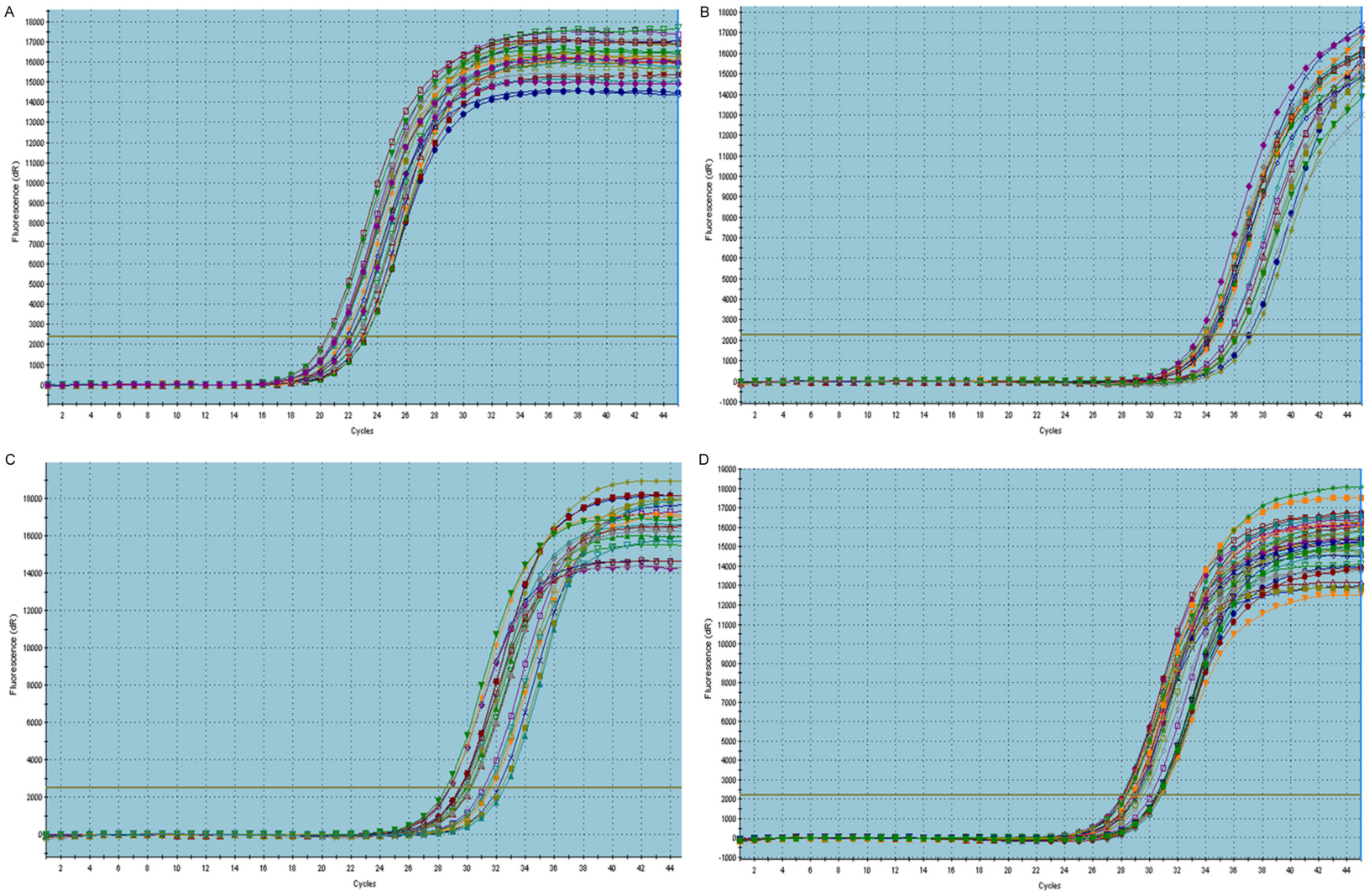
Levels of Ngn-2 mRNA in the control group increased rapidly from 12 hours after the start of differentiation but decreased rapidly after reaching a peak at day 1. Levels then decreased slightly after differentiation (**Table 2**). Levels of Ngn-2 mRNA in all infected groups were lower than the control group at day 1 ($P < 0.05$; **Table 2**), increasing gradually after reaching a minimum at day 2, then increasing gradually and reaching a peak at day 5 after the start of differentiation. However, its peak was still lower than that of the control group. Levels of Ngn-2 mRNA in the MOI = 5 group were lower than the MOI = 0.1 group from day 2 to 5 and in the MOI = 1 group at day 2 and from days 4 to 5 ($P < 0.05$; **Table 2**). Amplification curves of Ngn-2 are shown in **Figure 5D**.

Levels of c-Myc in the control group increased gradually and decreased slightly after reaching a peak at day 2 after the start of differentiation, ending at the maximum level (**Table 2**; **Figure 5E**). Levels of Ngn-2 mRNA in all infected groups were lower than the control group from day 1 to 4 ($P < 0.05$; **Table 2**). Levels in the MOI = 5 group were lower than the MOI = 0.1 group at 12 hours and from days 3 to 5 ($P < 0.05$; **Table 2**). Levels in the MOI = 1 group were lower than that of the MOI = 0.1 group at 12 hours and from days 3 to 4 ($P < 0.05$; **Table 2**).

Levels of cyclin D1 mRNA in the control group fluctuated after the start of differentiation. However, its range was small and reached a peak at day 2 (**Figure 5F**). Levels of cyclin D1 mRNA in all infected groups were lower than the control group from 12 hours to 1 day ($P < 0.05$; **Table 2**), and higher than the control group at day 3. Levels in the MOI = 5 group were lower at day 1 and from days 4 to 5 than in the MOI = 1 group, as well as higher at 12 hours and day 2 than in the MOI = 0.1 group ($P < 0.05$; **Table 2**).

Discussion

Congenital cytomegalovirus infection is a significant cause of brain disorders, including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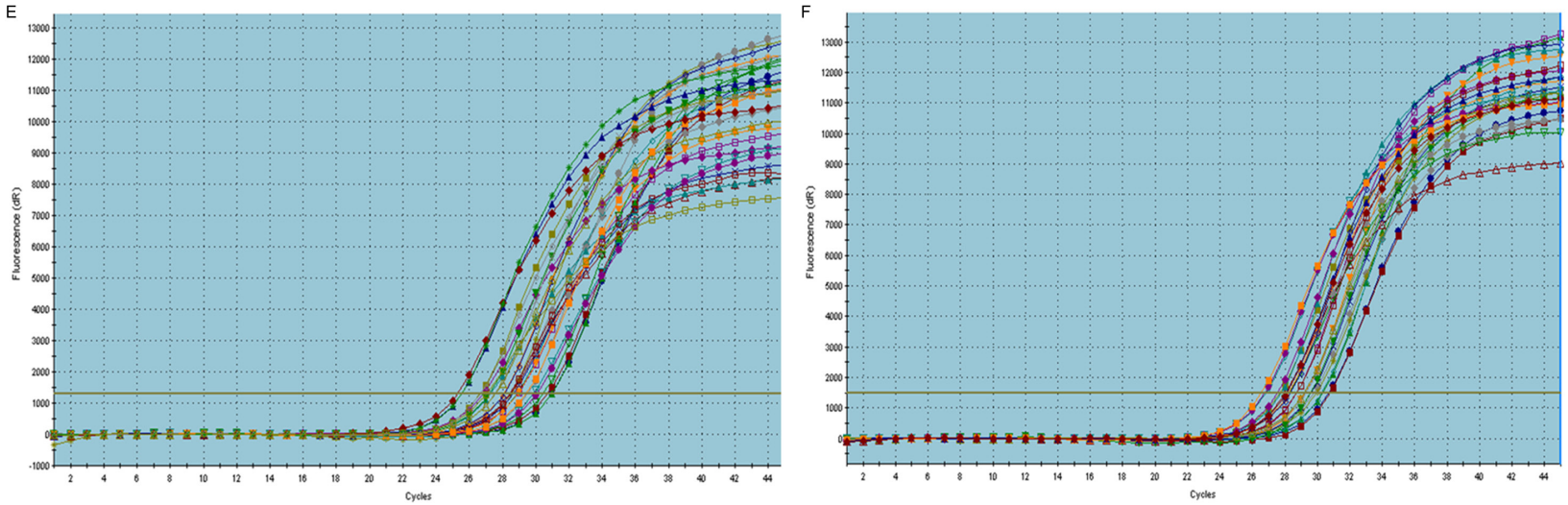

Figure 5. Amplification curves of differentiation genes. A. Amplification curves of GAPDH. B. Amplification curves of Wnt-3. C. Amplification curves of Wnt-7a. D. Amplification curves of Ngn-2. E. Amplification curves of c-Myc. F. Amplification curves of cyclin D1.

microcephaly, mental retardation, hearing loss, and visual disorders in fetuses [11]. However, the underlying mechanisms by which congenital cytomegalovirus infection exerts an influence on the central nervous system have remained elusive. Here, for the first time, this study demonstrated that MCMV infection significantly inactivates Wnt signaling pathways and inhibits NSPC cell differentiation in an *in vitro* cell culture model.

This study isolated cells from mouse embryonic brain tissue, culturing them under *in vitro* conditions. Forming neurospheres and expressing nestin protein, these cells could be passaged many times and differentiated into neurons and astrocytes, typical characteristics of NSPCs. In the *in vitro* NSPC system, the hypothesis that MCMV influences development and differentiation of NSPCs was tested.

With the help of an immunofluorescence study, morphological changes in cell cultures were observed for 3 successive weeks. NSPCs in infected groups were consistently round and single or conglobate without processes. They were completely different, morphologically, from cells in the control group. At all timepoints examined, fluorescence intensities of nestin in infected groups were higher than the control group. Accordingly, intensities of GFAP and NSE in infected cells were significantly lower than the control group. Differences were more obvious over time. These findings further suggest that differentiation of NSPCs might be inhibited by MCMV infection. Expression of MCMV early antigens could be detected in GFAP- or NSE-positive cells at each timepoint during differentiation of infected NSPCs. This indicates that neuroglial-like cells and neuron-like cells during differentiation and development of NSPCs may be more susceptible to MCMV infection.

Flow cytometry also revealed that rate of differentiation of NSPCs in infected groups was significantly changed by MCMV infection. This suggests that MCMV could inhibit differentiation of NSPCs into neurons and astrocytes and the effects are dose-dependent with MCMV MOI. However, mechanisms underlying the regulation of differentiation remain unknown. Therefore, this study employed real-time RT-PCR to detect expression of key upstream genes in Wnt signal pathways, Wnt-3, and Wnt-7a and key downstream genes Ngn-2, c-Myc, and cyclin D1. The expectation was to find involvement of these genes in the differentiation process of NSPCs at the early differentiation phase. It has been shown that proteins that were encoded by Wnt-3 and Wnt-7a genes can combine with Frz, LRP5 and LRP6 at the cell surface to activate Wnt signal pathways, transfer signals into the nucleus by β-catenin/TCF, regulate expression of its downstream target genes, Ngn-2, c-Myc, and cyclin D1, and induce differentiation of NSPCs into neurons and astrocytes. Therefore, they are differentiation-inducing factors of NSPCs [12-14].

Results of this study showed that expression levels of Wnt-3 and Wnt-7a mRNAs in the infected groups were lower than the control group from the first to the second day of differentiation. Moreover, decreased magnitudes of expression in infected groups were greater as MCMV MOI increased. This indicates that MCMV could inhibit expression of the two genes and that effects of MCMV MOI on expression may be dose-dependent.

Ngn-2 is a member of the basic helix-loop-helix (bHLH) gene family, a functional gene determining differentiation of NSPCs cell-lineage. It is expressed in neural precursor cells [15-17]. It was found that expression of *Ngn-2* mRNA in the control group increased by the end of the first day of differentiation and was time-dependent. This result was consistent with research reports described previously [15]. Expression of *Ngn-2* mRNA in infected groups were lower than the control group at the first day of differentiation, then increasing slightly. However, the maximal level of expression was still lower than that of the control group. This indicates that expression of *Ngn-2* mRNA in the infected groups was inhibited.

Promoters of c-*Myc* and *Cyclin D1* genes contain transcription factor (TCF) binding sites and can combine with β-catenin/TCF in Wnt signal pathways to activate gene transcription [18]. The latest research on the subject reports that overexpression of *c-Myc* induces differentiation of NSPCs into neurons with electrophysiological activity [19]. In the present study, it was found that expression of *c-Myc* mRNA of the infected groups was lower than that of the control group from days 1 to 4 of differentiation culture. Protein encoded by the *Cyclin D1* gene, known as *Bcl-1*, can directly activate cyclin-

dependent kinase in G1 phase and regulate cell cycle progression from G1 to S [20]. In the present study, expression of *Cyclin D1* mRNA in the infected groups was remarkably lower than the control group. This indicates that MCMV infection could influence cell cycle progression during differentiation and development of NSPCs by moderating Ngn-2 and cyclin D1.

Differentiation and development of NSPCs is a progressive and orderly process [21]. Neurons and astrocytes from NSPCs constitute the basis of cerebral tissue structure and physiological function during the embryonic period. This study's findings suggest that MCMV inhibits differentiation of NSPCs into mature nerve cells by suppressing expression of key upstream and downstream genes of Wnt signal pathways. Moreover, the inhibitory effects were dose-dependent with MCMV MOI. These findings may be closely related to anomalies of the central nervous system that have resulted from congenital CMV infection. One previous study has shown that the *M122* gene of MCMV is an initial and essential factor of its neural invasion process, in which the promoter of this gene plays a key role [22]. Therefore, this gene may be essential in the differentiation and development of NSPCs.

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Disclosure of conflict of interest

None.

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