# Human cytomegalovirus infection enhances cell proliferation, migration and upregulation of EMT markers in colorectal cancer-derived stem cell-like cells

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**Abstract.** Increasing evidence suggests a link between persistent human cytomegalovirus (HCMV) infection and cancer. Although the role of HCMV in cancer is still elusive, recent studies revealed the presence of HCMV nucleic acids and proteins in different cancer types such as glioblastoma, colorectal, breast, and prostate cancers, and neuroblastoma. Although HCMV may not be directly associated with the neoplastic transformation, the presence of HCMV DNA in the tumorous tissue has been associated with altered clinical outcomes in cancer patients. However, the mechanisms involved in the association between colorectal cancer (CRC) and HCMV are unclear. In this study, we investigated the influence of HCMV infection on CRC or their derived cells. Proliferation and migration assays revealed a high infection efficiency in CRC-derived HT29 and SW480 'stem-like' cells. After 24, 48 and 72 h of HCMV infection, both HT29 and SW480 parental and stem-like cells showed a significant increase in cell proliferation and viability ($p<0.0001$). Moreover, HCMV infection promoted cell migration. These results demonstrate a significant phenotypic alteration in the CRC cell line upon HCMV infection. Using epithelial to mesenchymal transition (EMT) assays, we demonstrated that the EMT markers and driver genes were upregulated during the virus infection. The WNT signaling pathway, which is associated with the proliferation and migration of CRC cells, was upregulated (6-fold) in HCMV-infected cells as compared to the non-infected cells at day 7 from infection.

## Introduction

According to the World Health Organization, colorectal cancer (CRC) is the third leading cause of cancer-related death in the world after lung and liver cancers. Of the 8.8 million deaths reported in 2015, 774,000 cases were attributed to CRC (1). There are at least four types of human colorectal carcinogenesis, namely adenoma-carcinoma, hereditary non-polyposis colorectal cancer (HNPCC), *de novo* cancer, and colitis cancer (2). Many cases of CRC are related to environmental or dietary factors rather than heritable genetic changes. These factors include the environmental and food-borne mutagens, specific intestinal commensals, pathogens, and chronic intestinal inflammation, which subsequently induce tumor development. The progression from adenoma to cancer and

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metastatic stage involves the reciprocal failure of protective mechanisms such as adenomatous polyposis coli (APC), p53, and transforming growth factor β (TGF-β) as well as the induction of oncogenic pathways such as K-RAS and β-catenin (3-6).

For the past decade, the development of CRC is seldom being linked to infectious diseases. However, recent studies showed that the proteins immediate early 1 (IE1) and pp65 of human cytomegalovirus (HCMV) were detected in colorectal polyps and adenocarcinomas but not the adjacent non-neoplastic colon biopsy samples (7). The presence of HCMV proteins, mRNA of early genes, and DNA was demonstrated through immunochemical staining, in situ hybridization, and polymerase chain reaction (PCR), respectively (7,8). In addition, our previous study reported the presence of HCMV nucleic acids in the tumorous epithelium of CRC. Furthermore, the existence of HCMV in CRC was correlated with the poor outcome in elderly group but better outcome in the younger group (8,9). Dimberg *et al* showed that the HCMV-DNA-positive rate was significantly higher in cancerous tissue as compared with the paired normal tissue (10). Growing evidence demonstrates that HCMV infection occurs in tumor tissues and its gene products may promote important oncogenic pathways in CRC (11).

Human cytomegalovirus belongs to the subfamily of β-herpesviruses. Upon infection, it gets adapted and remains lifelong in the host. The viral replication cycle is reactivated whenever the host immunity is impaired, resulting in disease relapse (12). HCMV comprises a genome of ~235 kb with >200 open reading frames (ORFs) that encode >180 proteins. Among these proteins, some are essential for its replication and a vast majority may interfere with the cellular and immunological functions to enable the virus to coexist with its host (13). Several studies provide evidence that HCMV proteins and nucleic acids are frequently detected in tissue specimens from patients with cancers of different origin, including cancer of colon (7,8-11), breast (14), prostate (15), and mucoepidermoid salivary gland (16) as well as glioblastoma (17-19) and neuroblastoma (20). In addition, HCMV proteins are believed to function as 'oncomodulators' in cancer. There have been a number of studies suggesting HCMV proteins such as IE, US28, pp65, non-coding RNA β 2.7kb (β 2.7 kb) and other transcripts enable the virus to provide mechanisms for oncomodulation, thus enable the virus to evade from host immune and aid in the oncogenic transformation (21-23). Some of the HCMV gene products and proteins are known to accelerate cancer progression via certain pathways. Some of these pathways are involved in the suppression of the local immune response against tumors, while others are involved in the promotion of cell proliferation, apoptosis, angiogenesis and metastasis.

Increasing evidence revealed HCMV infection in glioblastoma multiforme (GBM) and glioma stem cell (GSC), which are believed to cause the recurrence of GBM after the surgery or therapy (24-27). However, the impact of HCMV infection in CRC and developing tumors is questionable, especially in colon cancer stem cell (CSC). To date, there is no well establish cell model to study the interaction of HCMV and CRC. In this direction, we studied the influence and effect of HCMV in CRC-derived cell lines.

## Materials and methods

*Virus infection.* The laboratory-adapted strain of HCMV AD169 obtained from American Type Culture Collection (ATCC, USA) was propagated in confluent monolayers of MRC-5 cells (ATCC) in minimal essential medium (MEM) (Gibco, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA). Supernatants were harvested from MRC-5 cells displaying 90-100% cytopathic effects (CPE) and the aliquots were store at -80˚C. Infectious titers of all virus stocks were determined by performing the plaque assay on MRC-5 cells. Virus propagation was carried out by low multiplicity of infection (MOI).

*Cell culture.* HT29 and SW480 cells were provided by Professor Hsei-Wei Wang of National Yang-Ming University, Taiwan. HT29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies) and SW480 cells were cultured in Leibovitz's L-15 medium (Gibco, Life Technologies) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, Life Technologies). MRC-5 cells (ATCC) were cultured in MEM supplemented with 10% FBS. All cells were cultured at 37˚C with 5% $CO_2$.

*Sphere formation assays.* For stem-like culture, HT29 and SW480 parental cells were resuspended in serum-free DMEM/F12 medium supplemented with 1X N-2 supplement (Gibco, Life Technologies), 10 ng/ml recombinant human epidermal growth factor (EGF) (Sigma-Aldrich, USA), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich), and 1% penicillin/streptomycin. Cells were plated at a density of $10^2$, $10^3$ or $10^4$ cells/well, as per the experimental requirement, and monitored for 2-3 weeks until spheroids were formed.

*Flow cytometry analysis.* Flow cytometry assay was used to analyze the expression profile of the cancer stem cell marker CD44. Briefly, ~$10^6$ cells were washed with phosphate-buffered saline (PBS; Amresco, USA) and labeled with FITC-conjugated anti-CD44 (Miltenyi Biotec, Auburn, CA, USA) in the dark for 30 min at room temperature. Following incubation, cells were washed twice with PBS and analyzed using a Cytomics FC 500 Series flow cytometry system (Beckman Coulter, Indianapolis, IN, USA).

*Immunofluorescence assays and determination of the infectivity rate of HCMV in CRC derived cells.* To determine the HCMV infection in cells, immunofluorescence assays were carried out by labeling the non-infected and infected cells with cytomegalovirus immediate early (IE) antibody (GeneTex, USA) and anti-cytomegalovirus pp65 antibody (Abcam, USA). To determine viral infectivity, $10^3$ parental and stem-like HT29 cells were infected with HCMV AD169 at MOI of 5 on coverslips. After 24, 48 and 72 h of infection, infected and non-infected cells were fixed with ice-cold methanol for 10 min at room temperature and washed thrice with PBS. Cells were blocked with 1% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature, followed by three washes of PBS. Both infected and non-infected cells were stained with 1:20 cytomegalovirus IE antibody for 1 h in a humidified chamber at 37˚C. Following incubation, cells were washed thrice with

Table I. The primers used for RT-PCR and real-time PCR.

| Gene | Forward (5'-3') | Reverse (5'-3') |
|------|-----------------|-----------------|
| RT-PCR | | |
| IE1 | CGACGTTCCTGCAGACTATG | TCCTCGGTCACTTGTTCAAA |
| US28 | GTGAACCGCTCATATAGACC | GAAACAGGCAGTGAGTAACG |
| β 2.7 kb | AAGATGTTGCGATGCGGTTG | CGGTCAGCAGCCAAACAATC |
| GAPDH | ACCACAGTCCATGCCATCAC | TCCACCACCCTGTTGCTGTA |
| qPCR | | |
| IE1 | AAGCGGCCTCTGATAACCAAG | GAGCAGACTCTCAGAGGATCG |
| US28 | GTACCACAGCATGAGCTTTTC | GTATAATTTGTGAGACGCGACA |
| β 2.7 kb | AAGATGTTGCGATGCGGTTG | CGGTCAGCAGCCAAACAATC |
| Wnt 11 | GACACAAGACAGGCAGTG | GTCCTTGAGCAGAGTCCT |
| FZD7 | AAGACTTGCAGGACGATGCT | TGTATCTCCCACTCGCCTTC |
| CTNNB | GTGCTATCTGTCTGCTCT | CATCCCTTCCTGTTTAGTTG |
| GSK3β | AAGTTAGCAGAGACAAGGA | CGCAATCGGACTATGTTAC |
| GAPDH | CTGCCCCCTCTGCTGATG | TCCACGATACCAAAGTTGTCATG |

PBS and probed with an anti-mouse IgG FITC (GeneTex) secondary antibody (1:2,000 dilution) for 1 h in a humidified chamber at 37˚C. Cells were washed as described above and stained with 4',6-diamidino-2-phenylindole (DAPI). The washing step was repeated and a coverslip was placed on the slide with a mounting agent. The slide was visualized using a fluorescence microscope fitted with a camera for cell counting. Five low-magnification fields were counted for each condition and the percentage of positive cells was calculated by dividing the number of IE-positive cells with the total number of nuclei, followed by multiplication with 100.

*Cell viability and cell proliferation*. Cell proliferation was evaluated in triplicates by a colorimetric WST-1 assay. The assay determines cellular viability by measuring the metabolic conversion of a water-soluble tetrazolium salt into a dark red formazan by mitochondrial dehydrogenases. The amount of formazan produced is proportional to the number of live cells, which is expressed as cellular viability. Briefly, $10^2$ non-infected and infected cells were seeded in 96-well plates and incubated for 6, 12, 24, 48 and 72 h. The assay was performed by adding WST-1 (Roche, Germany) directly to culture wells, followed by incubation for 1 h at 37˚C. Plates were read using DS2® (Dynex, USA) by measuring the absorbance of the dye at 450 nm wavelength, with 620 nm set as a reference wavelength. Each experimental condition was performed in triplicates.

To determine the growth effect of HCMV on infected cells, the cell proliferation was evaluated by direct cell counting. HCMV infected and non-infected cells were seeded at a density of $10^4$ cells/cm$^2$ in 24-well plates and cultured for 3, 6, 12, 24, 48 and 72 h. Following incubation, cells were washed with PBS and harvested by trypsinization. The cell number was determined following staining with 0.4% trypan blue by Countess Automated Cell Counter (Invitrogen/Life Technologies). The experiment was repeated thrice and each reaction condition was performed in triplicates.

*Migration assays*. For Transwell migration assays, dissociated stem-like or adherent parental HT29 and SW480 cells infected or non-infected with AD169 were plated at $10^3$ cells/cm$^2$ on the top chambers containing non-coated membrane with 8 µm pore size (Corning, NY, USA). Cells in the top chamber were grown in 100 µl serum-free medium, while the lower chamber was filled with 600 µl of 10% FBS-supplemented DMEM/F12. After 6, 12, 24, 48 and 72 h of infection, cells on the upper side were removed and those under the surface were fixed and stained with crystal violet. The cell number was counted using a microscope at five magnification fields. All assays were performed in triplicates.

*Reverse transcription (RT)-PCR and real-time PCR (quantitative PCR)*. RNA was extracted using TRIzol (Sigma-Aldrich) and 2 µg of RNA from each sample was used for the synthesis of the complementary DNA (cDNA). Reverse transcription was carried out in a reaction containing 2 µl of 10X RT buffer, 2 µl of 10X RT random primers, 0.8 µl of 100 mM dNTP mix, 1 µl of MultiScribe™ reverse transcriptase, 1 µl of RNase inhibitor (ABI, USA), and diethyl pyrocarbonate (DEPC)-treated water (total volume of 10 µl). Following reaction, 2 µg of total RNA in a total volume of 10 µl was added to the master mix. The reverse transcription condition was as follows: 25˚C for 10 min and 37˚C for 120 min, heat inactivation at 85˚C for 5 min, and cooling on ice. The cDNA synthesized was stored at -20˚C until its use for PCR. Real-time PCR reaction was performed by mixing 12.5 µl 2X SYBR master mix (ABI), 2 µl cDNA, 0.25 µl primer pair mix (0.1 µM/µl each primer), and 23 µl water with the PCR reaction. The PCR cycle was as follows: 1 cycle at 50˚C for 2 min, 1 cycle at 95˚C for 10 min, 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec, 72˚C for 30 sec, and a final extension at 72˚C for 10 min. Real-time PCR was performed in CFX Connect™ Real-Time Detection System (Bio-Rad, USA) and the results were analyzed with the CFX Manager™ Software (Bio-Rad). Gene expression level was normalized with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the fold change was calculated as $2^{-\Delta\Delta Ct}$, which is the normalized gene expression $2^{-\Delta Ct}$ in the infected sample divided by the normalized gene expression $2^{-\Delta Ct}$ in the non-infected sample.
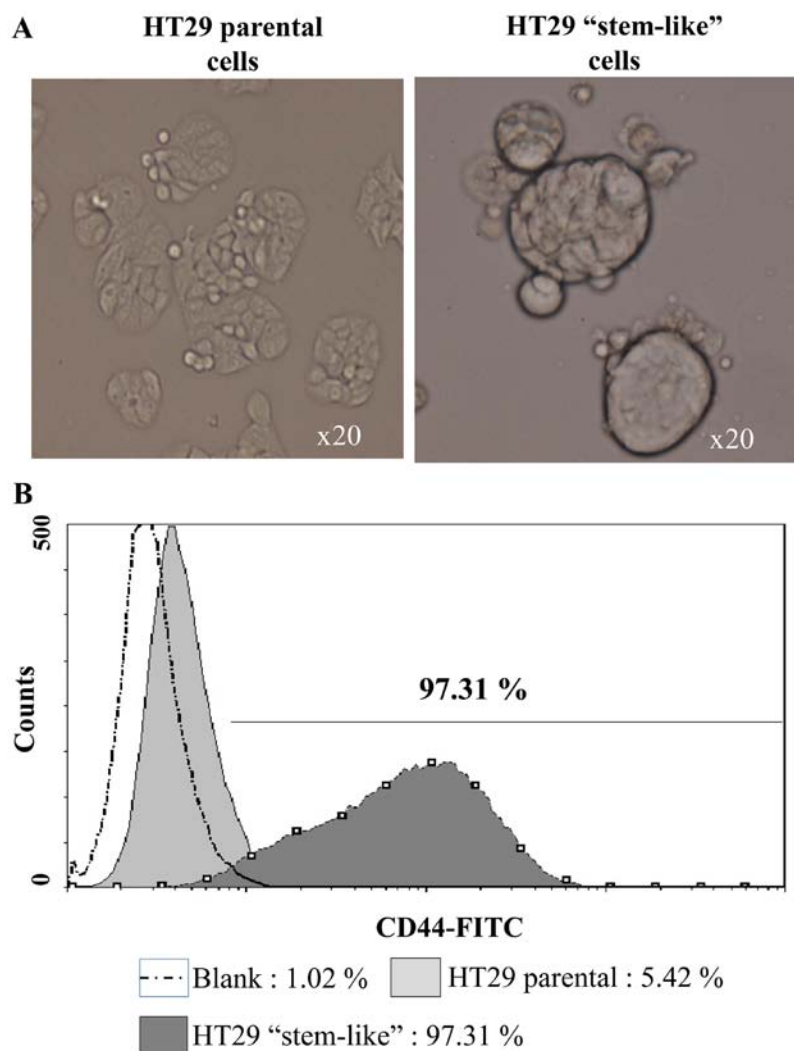
Figure 1. Generation of sphere from HT29 parental. (A) HT29 parental and stem-like cells. (B) Determination of the stemness marker CD44-FITC.

For RT-PCR, the PCR mixture was prepared in a total volume of 25 µl and included 2X Taq PLUS PCR Smart mix 1 (SolGent™, Korea) and the forward and reverse primers at a concentration of 0.3 µM each. The volume was adjusted with DEPC-treated water. PCR was performed with an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final elongation step for 4 min at 72°C. The primers used in this study are summarized in Table I.

*Analysis of epithelial to mesenchymal transition (EMT) pathways*. HCMV AD169 was used to infect $10^6$ HT29 stem-like cells at MOI of 5. Cells were harvested and total RNA extracted with TRIzol. The extracted total RNA was sent to Genomics, Taiwan, for human epithelial to mesenchymal transition (EMT) RT™ Profiler™ PCR Array (SABiosciences/Qiagen, Germany) analysis. Briefly, the total RNA was reverse-transcribed and the resulting cDNA analyzed with a 96-well plate quantitative PCR array. The array includes 84 key genes of EMT signal pathways. Gene expression levels were quantified and analyzed with the vendor's web-based software module. Data were collected and normalized based on the mean Ct value from five housekeeping genes in the arrays (ACTB,

B2M, GAPDH, HPRT1 and RPLP0) and further normalized to the untreated control sample. For fold-change comparisons in cells, non-infected cells were used as the control sample. The fold-change of gene expression was calculated as $2^{-\Delta\Delta Ct}$, which is the normalized gene expression $2^{-\Delta Ct}$ in the infected sample divided by the normalized gene expression $2^{-\Delta Ct}$ in the non-infected sample.

*Statistical analyses*. Data shown represent results of two independent experiments, with each reaction performed in triplicates. Statistical analyses were carried out by the two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test using GraphPad Prism software to compare between groups. We used the Student's t-test to evaluate two independent experimental groups. A value of $p < 0.05$ was considered as statistically significant.

## Results

*Increased HCMV infectivity in CRC derived stem-like cells*. We assessed HCMV infection in CRC using parental and stem-like HT29 cells as models (Fig. 1A). The enriched HT29 stem-like cells were analyzed using the CD44 marker.
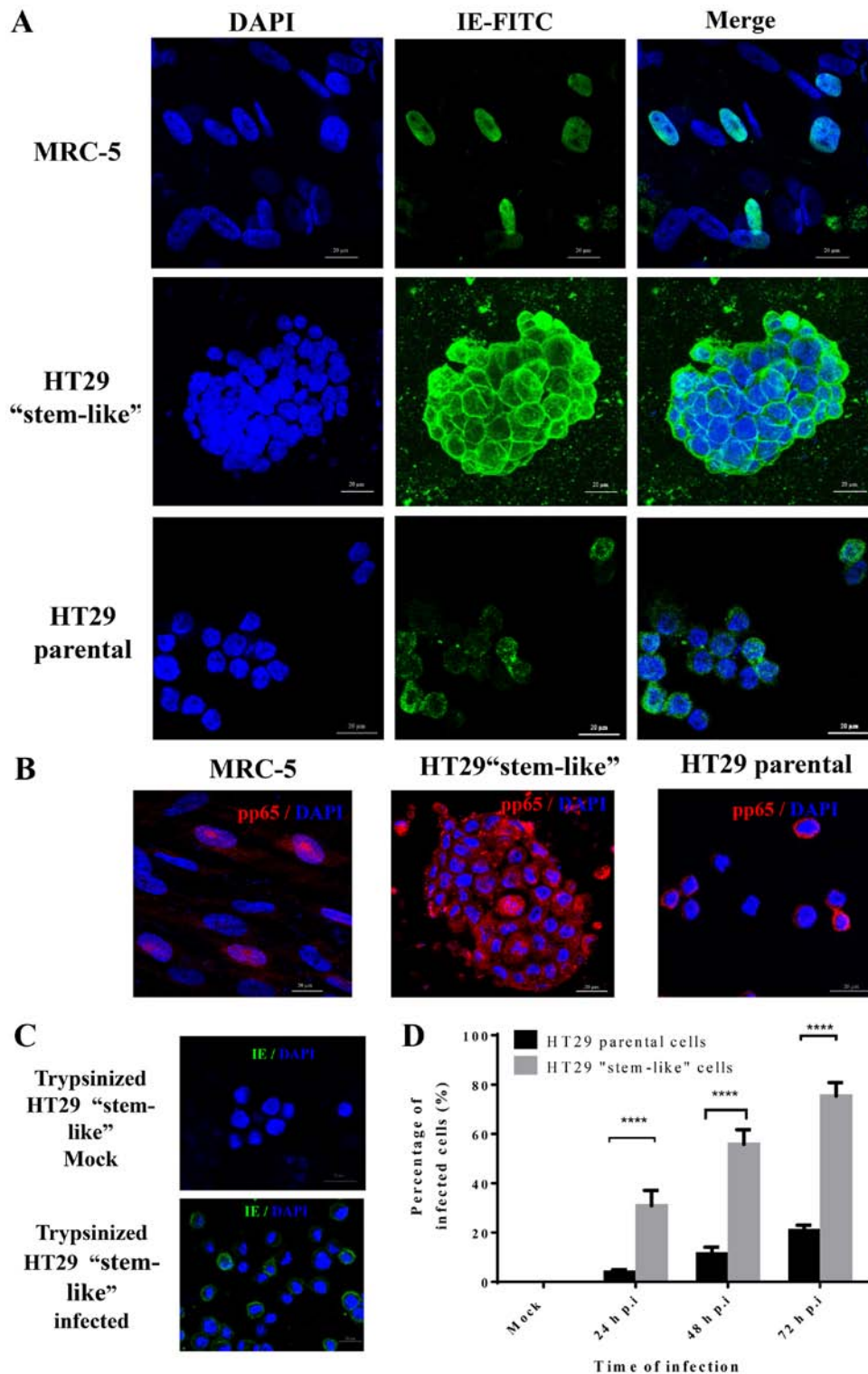
Figure 2. The infection of HCMV laboratory strain AD169 in colorectal cancer-derived cells. (A) HCMV AD169 was used to infect $10^3$ parental and stem-like HT29 and MRC-5 (control) cells at MOI of 5 on coverslips, followed by IE and (B) pp65 immunofluorescence. (C) IE-positive cells were observed after counter staining using a fluorescence microscope fitted with a camera. Five low magnification fields were counted for each condition. (D) The percentage of positive cells was calculated by dividing the number of IE-positive cells with the total number of nuclei, followed by multiplication with 100. ****$p<0.0001$ by two-way ANOVA and Tukey's multiple comparisons test.

Flow cytometry data showed that CD44+ cell population was significantly higher (97.31%) in the HT29 stem-like cells as compared with the parental HT29 cells (5.42%) (Fig. 1B). Both cell types were infected with the laboratory strain AD169 and the infection was confirmed by immunofluorescence staining with CMV IE and pp65 (Fig. 2A and B). Infection efficiency was defined as the number of IE-positive cells divided by the total number of nucleated cells. We observed a significantly large number of HT29 stem-like cells infected with AD169 as compared with the HT29 parental cells. To facilitate the
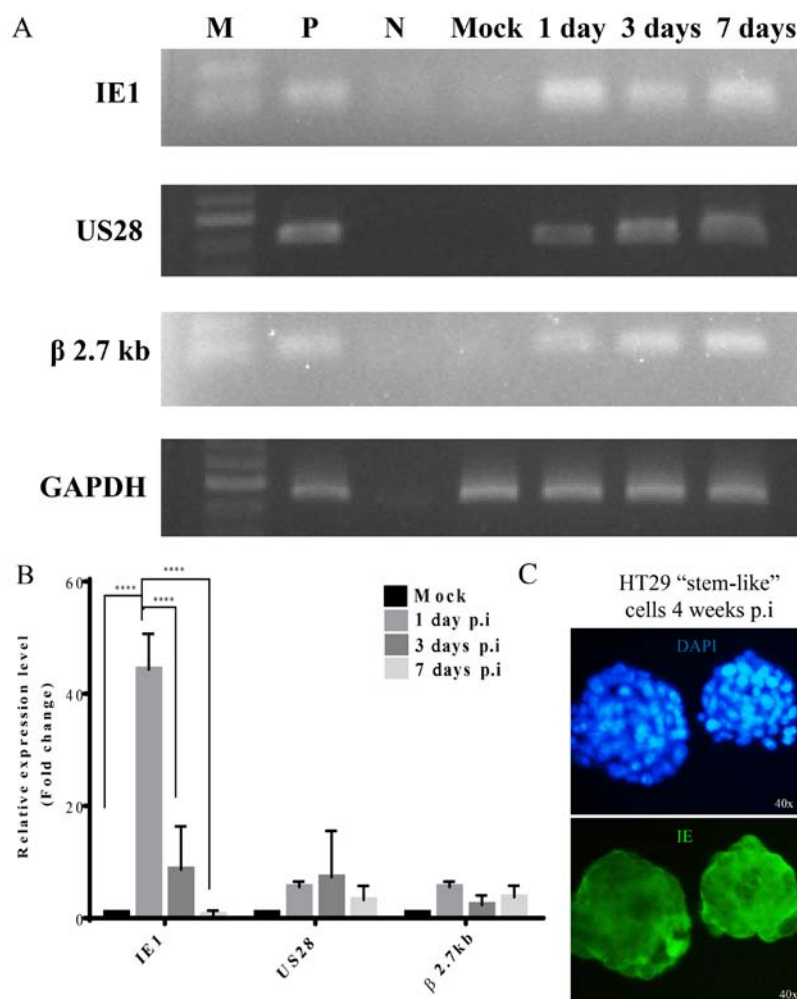
Figure 3. HCMV gene expression. (A) RT-PCR detection of IE1, US28, and β 2.7 kb transcripts in HACMV AD169-infected HT29 stem-like cells. M, 100 bp DNA ladder; P, positive control; and N, negative control. (B) Semi-quantitative SYBR Green-based RT-qPCR of IE1, US28, and β 2.7 kb at different time-points following HCMV AD169 infection of HT29 stem-like cells. ****p<0.0001 by two-way ANOVA and Tukey's multiple comparisons test. (C) Determination of HCMV AD169 long-term infection in HT29 stem-like cells (4 weeks).

HT29 stem-like cells count, the infected spheroid cells were dispersed by trypsinization (Fig. 2C). After 24-h infection, IE-positive cells were ~3% in HT29 parental cells as compared to 29% in HT29 stem-like cells. After 72-h infection, ~70% of HT29 stem-like cells were IE-positive as compared to only 20% IE-positive HT29 bulk cells (Fig. 2D).

*HCMV gene expression.* To evaluate the mechanism of HCMV infection in HT29 stem-like cells, we infected these cells with AD169 at MOI of 5 and determined the expression pattern of HCMV genes at different time post-infection using RT-PCR and qPCR. RT-PCR data showed different HCMV gene expression (Fig. 3A). Following AD169 infection, the HCMV IE1 transcript was detected at 24 h, but it decreased at 72 h and day 7. A small increase in the expression of US28 late gene and β 2.7 kb was reported at 24 h, 72 h, and day 7 after infection.

We used SYBR Green real-time PCR for accurate quantification of the mRNA expression of HCMV genes. Following infection, IE1 expression surged at day 1 but decreased at day 3 and 7 (Fig. 3B), while the IE1 mRNA level decreased at all time-points. On the other hand, no significant differences in US28 and β 2.7 kb mRNA expression levels were observed after day 1, 3 or 7 of HCMV infection. In HT29 stem-like cells with 4 weeks prolonged AD169 infection, we found that the IE proteins were still expressed (Fig. 3C).

*Cell proliferation and viability.* We used the colorimetric WST-1 assay to evaluate the viability of HT29 and SW480 cells after AD169 infection. HT29 and SW480 parental and stem-like cells infected with AD169 at different time-points along with the non-infected control were prepared in triplicates. We observed increased cell viability in HT29 and SW480 stem-like cells infected with AD169 (Fig. 4A and B). In comparison to the non-infected cells, HT29 stem-like cells infected with AD169 showed a significant increase in cell proliferation after 12 (0.96±0.05, p=0.011), 48 (3.10±0.04, p<0.001), and 72 h (3.31±0.03, p<0.0001) of infection. Besides, we also observed a significant increase in SW480 stem-like cells infected with AD169 after 24 (1.49±0.02, p<0.0001), 48 (2.79±0.06, p<0.0001), and 72 h (3.10±0.04, p<0.0001) compared to the non-infected cells. No significant growth difference was observed between the HT29 and SW480 parental cells infected with AD169 and non-infected cells.
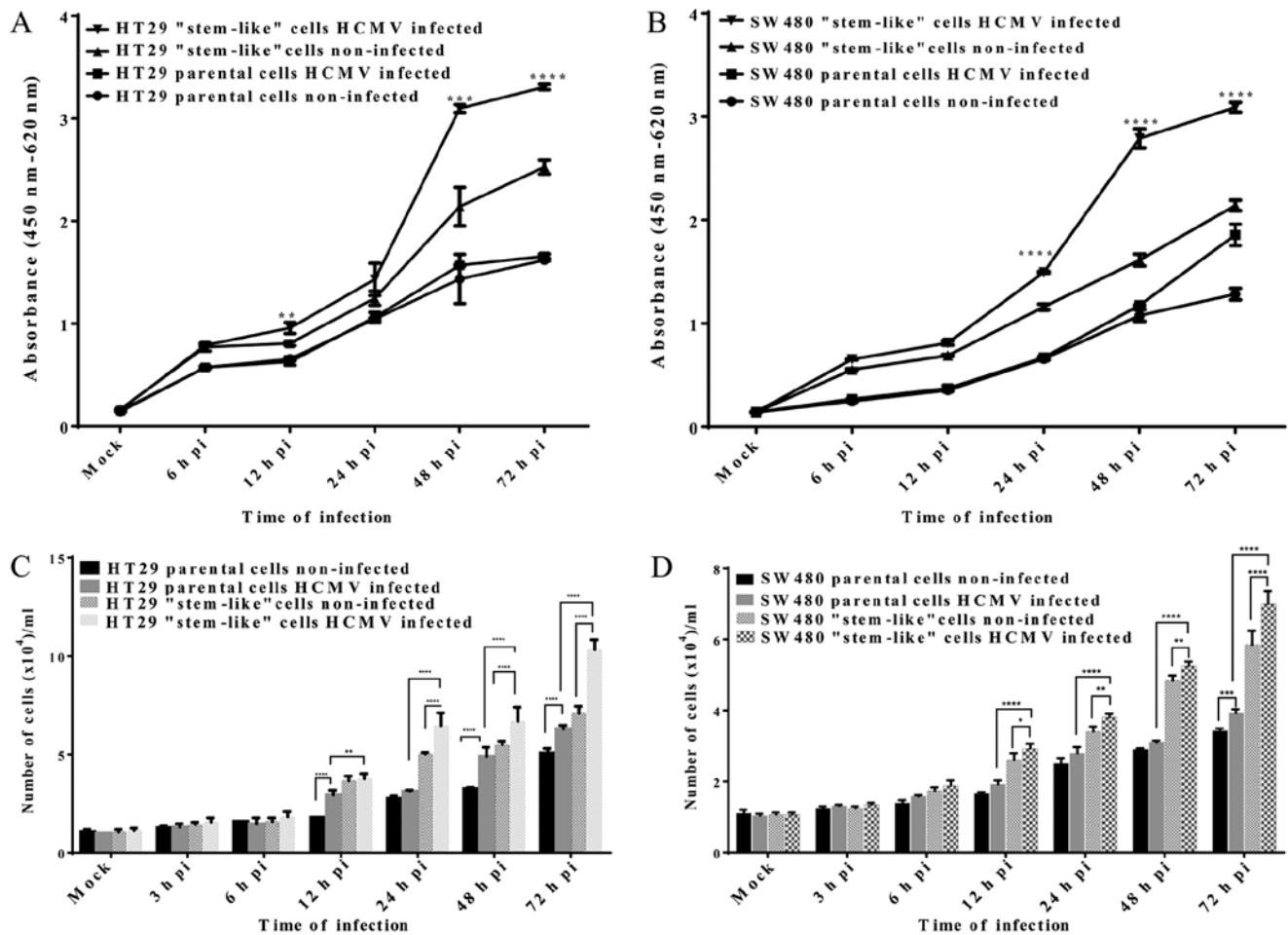
Figure 4. Cell viability and proliferation of AD169-infected and non-infected colorectal cancer-derived cells. HCMV AD169 was used to infect $10^2$ parental and stem-like (A) HT29 cells and (B) SW480 cells at MOI of 5. Cells were harvested at 6, 12 24, 48 and 72 h following infection. Cells were subjected to WST-1 assays. AD169-infected cells showed significant proliferation compared to the non-infected cells. $^{**}p < 0.01$, $^{**}p < 0.001$ and $^{***}p < 0.0001$ by Student's t-test. For direct cell proliferation evaluation, $10^4$ parental and stem-like (C) HT29 cells and (D) SW480 cells were infected with AD169 at MOI of 5. Cells were harvested at 3, 6, 12 24, 48 and 72 h following infection and stained with 0.4% trypan blue. Cells were counted using the Countess Automated Cell Counter. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$ by two-way ANOVA and Tukey's multiple comparisons test.

We evaluated the proliferation of AD169-infected cells by direct cell counting. HT29 and SW480 parental and stem-like cells infected with AD169 at different time-points were harvested, stained with trypan blue, and counted using the Countess Automated Cell Counter. The results revealed that AD169 infection promoted cell growth. Both HT29 and SW480 parental and stem-like cells showed a significant increase in growth following infection (p<0.001) (Fig. 4C and D). However, the growth rate was higher for the infected HT29 or SW480 stem-like cells as compared to the infected parental cells at all time-points. In comparison to the infected parental cells, infected stem-like cells showed a 1.3, 2.1, 1.4, and 1.6-fold increase in the growth rate after 12, 24, 48 and 72 h of infection, respectively. The infected HT29 stem-like proliferated at a significantly faster rate compared either with the non-infected HT29 stem-like or HT29 infected and non-infected parental cells. We observed that SW480 infected stem-like cells also proliferated faster than the parental infected cells. The growth rates after 12, 24, 48 and 72 h of infection were 1.5, 1.4, 1.7 and 1.8-fold higher than those of the parental infected cells. These data suggest that HCMV infection may promote cell proliferation.

*HCMV infection increased cell migration ability.* We used Transwell migration assays to study the migration ability of HT29 and SW480 cells with or without AD169 infection. In comparison to the non-infected cells, the infected HT29 and SW480 parental and stem-like cells showed significantly higher migration ability (p<0.001). However, the number of migrated cells was more in the stem-like cells as compared with the infected parental cells (Fig. 5). In addition, the number of migrated SW480 infected stem-like cells was higher than that of HT29 infected stem-like cells. Thus, HCMV infection increased the migration ability of colorectal-derived cells.

*EMT RT² PCR analyses.* To investigate the involvement of EMT in the increased migration ability of these cells, we evaluated the expression of EMT-associated genes in infected and non-infected HT29 stem-like cells using the RT² Profiler™ PCR array. The heat map (Fig. 6A) showed that most of the EMT-related genes were upregulated after HCMV infection. After 24-h infection, 62 genes were upregulated and 27 genes, downregulated. On the other hand, 35 and 57 EMT-related genes were upregulated and 49 and 27 genes were downregulated after 72 h and 7 days of infection, respectively.
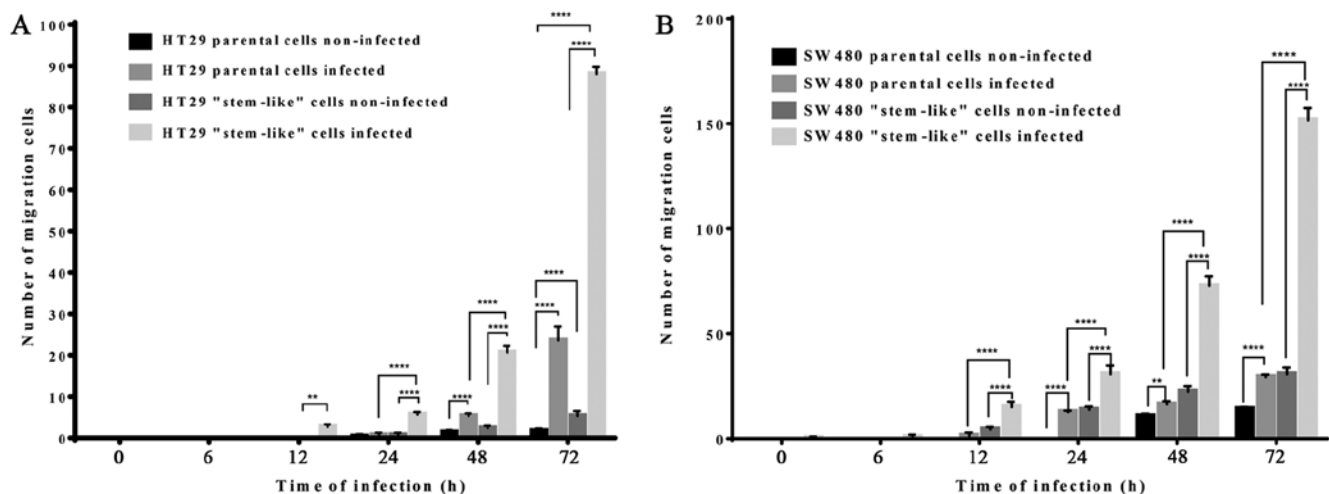
Figure 5. Migration assay of AD169-infected and non-infected cells. Parental and stem-like (A) HT29 cells and (B) SW480 cells were treated with or without HCMV AD169 at MOI of 5. After 72 h of infection, $10^4$ cells were translocated in a 24-Transwell chamber with non-coated membrane and incubated for 6, 12 24, 48 and 72 h. The cells under the membrane were stained with crystal violet and counted under the microscope. $^{***}$p<0.001 and $^{****}$p<0.0001 by two-way ANOVA and Tukey's multiple comparisons test.

The array data indicated an increase in the expression of mesenchymal markers such as N-cadherin and fibronectin at all time-points. On the other hand, E-cadherin was downregulated across the infection time. In addition, the expression of EMT drivers such as SNAIL1, SNAIL2/SLUG, ZEB1, and TWIST1 was upregulated following infection (Fig. 6B). We observed an increase in the level of WNT11, frizzled-7 (FZD7), glycogen synthase kinase 3β (GSK3β), and β-catenin (CTNNB1) during HCMV infection (Fig. 6C). In the subsequent analysis, we designed a panel of primers against WNT signaling (Table I) and evaluated the expression of WNT11, FZD7, GSK3β, and β-catenin by real-time PCR. As shown in Fig. 6D, we observed that the results were compatible with the results of the array.

In comparison to the non-infected cells, those infected showed a significant increase (6-fold) in the expression of WNT11 at day 7 following infection (p<0.001). The expression of FZD7 in the infected cells was 2.2±0.94-fold higher than that in the control cells. No significant different in GSK3β expression was noted. Although there was an increase in the expression of β-catenin during infection, the difference was not statistically significant.

## Discussion

In this study, we demonstrated that the HCMV strain AD169 infected HT29 stem-like cells with higher efficiency than the HT29 parental cells. The infection rate increased in a time-dependent manner. This result was consistent with a previous study, wherein the efficiency of HCMV infection was higher in 387 and 3832 GSCs as compared with the standard glioma cell line U87 and T98G (26). Fornara *et al* found that glioblastoma cells infected with HCMV exhibited the ability to grasp those GSC from differentiated condition, thereby enhancing the stem cell phenotype. Consequently, there was an increase in the number of GSCs (28). CSCs have been implicated in the colon carcinogenesis, however their existence had not been experimentally demonstrated until recently. Due to the complexity of their biology and

technical problems, the definite identification and isolation is still under debate (29,30). The cell adhesion molecule CD44 was one of the proposed CSC markers. CD44-positive cells seem to exhibit CSC properties, such as a single cell could form a sphere and a xenograft tumor that resembled the original lesion (31). Therefore, in this study we used CD44 as the CSC marker to verify the enriched tumor sphere HT29 cells. Du *et al* (31) showed that the CD44 was expressed at the bottom of the crypt in colon tissues where the stem cells are distributed as reported (32). Previously, we showed that HCMV viral nucleic acids were mostly found localized at the basal layer of crypt (8) and in this study, we demonstrated that the infection rate was higher in the HT29 stem-like cells. This suggests that HCMV may favor cancer stem cell-like cells for infection.

In the present study, we observed that the patterns of HCMV IE and pp65 localization in HT29 stem-like cells were different from the permissive cell MRC-5. In MRC-5 infected cells, IE and pp65 were expressed in the nucleus while in HT29 stem-like cells they were detected in both nucleus and cytoplasm, but mostly in the cytoplasm. In previous study, the β2.7 kb RNA was detected in both intranuclear and cytoplasmic human fibroblasts, but in non-permissive cells, the early transcript is only present within the nucleus (33). In this case, the HT29 stem-like cells might behave as fibroblasts, permissive cells. As showed in this study, HT29 stem-like cells infected with HCMV upregulated TWIST and SNAIL expression and enhanced EMT by downregulating E-cadherin and upregulating the N-cadherin, fibronectin and vimentin (34,35). As anticipated, the resulting cells may acquire fibroblast-like properties. Further study should be carried out to clarify this topographic pattern. We observed a different trend in gene expression at all time-points (Fig. 3A and B). The expression of IE1 gene decreased over time, while expression of US28 and β 2.7 kb transcripts showed fluctuations, which may be attributed to the different stages in the virus life cycle. HCMV has an organized genome expression. Its replication starts with the immediate early
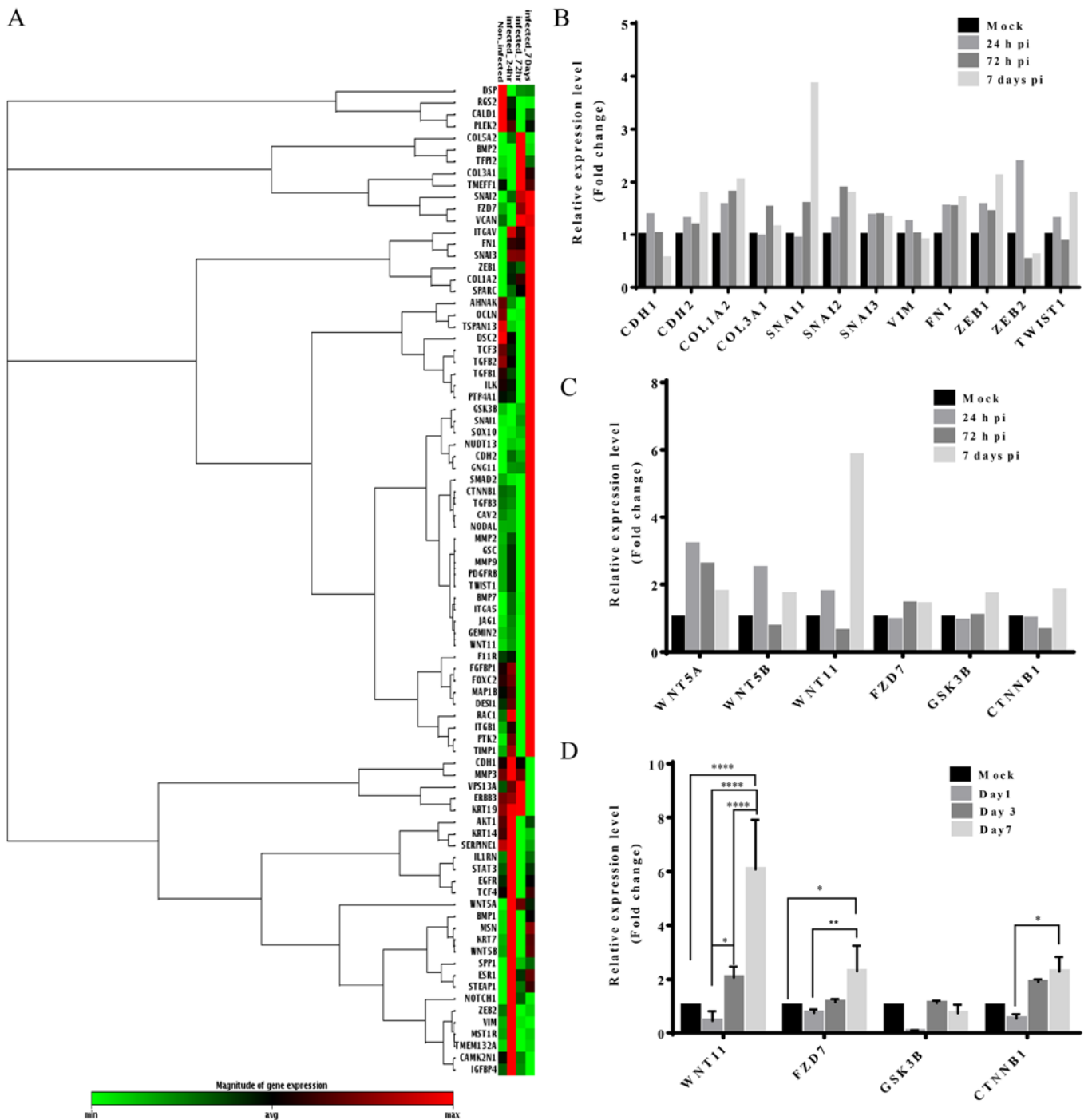
Figure 6. Human EMT RT² Profiler PCR Array of AD169-infected and non-infected cells. (A) HT29 stem-like cells were treated with or without HCMV AD169 at MOI of 5. After different time-points, cells were harvested and the total RNA was extracted and converted to cDNA. The hierarchical clustering of gene signatures was determined using RT² Profiler PCR Array of EMT and illustrated as heat maps (cut off value >2). (B) The expression of EMT markers and drivers gene of HT29 stem-like cells infected with AD169. (C) Expression of genes related to the WNT signaling pathway in HT29 stem-like cells infected with AD169. (D) Confirmation of WNT11/FZD7 expression in HT29 stem-like cells infected with AD169 and non-infected cells. *p<0.05, **p<0.01, and ****p<0.0001 by two-way ANOVA and Tukey's multiple comparisons test.

gene expression, followed by the expression of early and late genes. IE1, expressed in the initial phase, regulated the expression of other viral genes such as US28 and β 2.7 kb. The fluctuation in gene expression reported at certain time-points may be a signaling change in the virus replication (36-38).

In this study, we used two CRC-derived cell lines, HT29 and SW480 cells to verify the proliferation after AD169 infection. We found that either HT29 or SW480 infected stem-like

cells proliferated more than the non-infected cells or the infected parental and non-infected parental cells. In line with our finding, Fiallos et al, showed that a long-term infection of HCMV in GSC also promoted cell proliferation (26). At the same time, Fornara et al, claimed that the HCMV IE expression induced GBM cells to display stem-like phenotypes and promoted the growth of glioma cancer stem cells (GCSCs) (28). Significant proliferation was shown in the stem-like cells. This

is consistent with previous clinical finding in our group where the presence of HCMV in CRC patients with stage II, III and IV had a poor outcome (9,39). The tumoral presence of HCMV was associated with a decreased disease-free survival and this might due to the recurrence of CRC. The dysregulation of cell growth signaling in cancerous cells sustains chronic proliferation. Phosphatidylinositol-3-kinase/protein kinase (PI3K/AKT) and mitogen-activated protein kinase (MAPK) activation as well as phosphatase and tensin homologue (PTEN) mutation is known to promote tumorigenesis, as these signaling events stimulate growth, proliferation, and survival of cancer cells (40-43). It had been reported that HCMV gB induced activation of platelet-derived growth factor receptor α (PDGFRα) and PI3K/AKT, which increased growth and promoted survival and motility in cancer cells (44). HCMV IE proteins have been shown to induce expression of nuclear factor κB (NF-κB) subsequently activating the cell survival pathways in tumor cells (45). HCMV IE1 and IE2 were shown to interact with p53 suppressor to prevent the infected cells from undergoing cell growth arrest and apoptosis (46). In addition, HCMV encoded the protein pUL38, which mimics the mammalian target of rapamycin complex-1 by blocking the function of tuberous sclerosis protein 2 (TSC2). Inhibition of TSC2 by pUL38 dysregulates the mTOR pathway and induces survival signal in infected cells (47). Furthermore, Reeves *et al*, showed that β 2.7 kb interacts with the mitochondrial respiratory chain complex I in neuronal U373 cells and prevents cell death (23).

Accumulated evidence indicates that the HCMV protein US28 is one of the potential proteins that play an important role in tumor progression. US28 was found to induce an invasive and angiogenic phenotype in GBM. US28 has the ability to promote cell growth and induce progression of cell cycle and expression of vascular endothelial growth factor, a proangiogenic factor in NIH3T3. In intestinal cells, US28 was shown to activate β-catenin by inhibiting GSK3β. At the same time, it dysregulated the WNT signaling target genes such as *cyclin D*, *survivin* and *c-myc*, which are important for controlling cell proliferation (48-53). Furthermore, US28 promotes cell migration through the chemokines RANTES and monocyte chemoattractant protein 1 (MCP-1) (48). Another study showed that the interaction of integrin αvβ3 and PDGFRα with glioma cells resulted in an increase in the cell migratory ability (54). This explains the phenomenon observed in our study, wherein the infected colorectal cells showed greater migration ability.

Studies have proposed that the EMT pathway drives the progression of cancer to an aggressive metastatic stage. During the metastatic stage, tumor cells lose their adhesiveness with the adjacent cells, become more invasive, and develop cancer stemness properties. Several signaling pathways such as WNT/β-catenin, Notch, TGF-β, hedgehog, and EGFR are involved in the EMT process (55-60). In our study, we found that the expression of mesenchymal markers in EMT such as N-cadherin and fibronectin were increased upon infection with HCMV. On the other hand, E-cadherin was suppressed during HCMV infection. E-cadherin functions as a mediator during cell-cell adhesion and the loss of E-cadherin is known to induce EMT. β-catenin is thought to be secluded in E-cadherin adherent junction. APC malfunction in CRC may result in

GSK3β inhibition by the WNT signaling pathway, leading to the accumulation of β-catenin in the cell cytoplasm. This will induce the expression of target genes such as *c-myc* and *cyclin D* by the TCF/LEF-1 family transcription factors. Activation of these genes usually stimulates tumor progression (61-64).

The array data show that the expression of WNT11 was high during the infection time course. Therefore, we repeated the experiment by evaluating the expression of related genes such as WNT11, FZD7, GSK3β, and β-catenin. It was confirmed that the expression of these genes was higher in the infected cells as compared with the non-infected cells. Previous studies (65,66) revealed that either the canonical or non-canonical WNT signaling was implicated during the dynamic and reversible EMT and mesenchymal-epithelial transition during CRC progression. The high expression of WNT11 and its ligand FZD7 is reported to enhance proliferation and migration/invasion activities in the colon cancer cells (67,68). These findings explain the phenotypic changes observed in our study, wherein HCMV infection enhanced the proliferation and migratory ability of cells. This study successfully established a CRC culture model with high HCMV infectivity to facilitate investigation of the underlying mechanisms. Further studies are needed to identify the HCMV genes involved in these phenotypic changes.

## Acknowledgements

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