



## RESEARCH ARTICLE

# Multipotent mesenchymal stromal cells are fully permissive for human cytomegalovirus infection

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**Congenital human cytomegalovirus (HCMV) infection is a leading infectious cause of birth defects. Previous studies have reported birth defects with multiple organ maldevelopment in congenital HCMV-infected neonates. Multipotent mesenchymal stromal cells (MSCs) are a group of stem/progenitor cells that are multi-potent and can self-renew, and they play a vital role in multi-organ formation. Whether MSCs are susceptible to HCMV infection is unclear. In this study, MSCs were isolated from Wharton's jelly of the human umbilical cord and identified by their plastic adherence, surface marker pattern, and differentiation capacity. Then, the MSCs were infected with the HCMV Towne strain, and infection status was assessed via determination of viral entry, replication initiation, viral protein expression, and infectious virion release using western blotting, immunofluorescence assays, and plaque forming assays. The results indicate that the isolated MSCs were fully permissive for HCMV infection and provide a preliminary basis for understanding the pathogenesis of HCMV infection in non-nervous system diseases, including multi-organ malformation during fetal development.**

**KEYWORDS** human cytomegalovirus (HCMV); multipotent mesenchymal stromal cells (MSCs); susceptibility; umbilical cord; Wharton's jelly

## INTRODUCTION

Multipotent mesenchymal stromal cells (MSCs) are a group of adult stem cells with self-renewal ability and multi-lineage differentiation potency, and they are found in many tissues, such as bone marrow, adipose tissue, and peripheral blood (Young et al., 1995; Wei et al., 2013). MSCs have the potential to differentiate into various lineages (including adipocytes, osteoblasts, chondrocytes, and myoblasts) (Dominici et al., 2006) and are capable of migrating to tissue injury sites to prevent de-

leterious remodeling and improve recovery (Pittenger and Martin, 2004). Notably, MSCs have been shown to induce a tolerant immune cell phenotype by altering their inflammatory cytokine secretion pattern and decreasing immune recognition, which could possibly reduce the possibility of developing graft-versus-host disease (Aggarwal and Pittenger, 2005). Therefore, MSCs are generally used for tissue engineering applications (tissue repair or regeneration) and as a carrier cell in gene therapy, and numerous studies have examined their capability to repair damage to myocardial tissue, bone, tendon, cartilage, and meniscus (Pittenger et al., 1999).

Human cytomegalovirus (HCMV), a  $\beta$  human herpesvirus, represents the major infectious cause of birth defects, as well as an important pathogen for immunocompromised individuals. After primary infection, HCMV persists latently in the immunocompetent population but leads to significant morbidity and mortality in immunocompromised individuals (such as patients with AIDS and those who underwent solid organ transplantation)

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and neonates (Bale, 1984; Landolfo et al., 2003). From 5% to 10% of congenitally HCMV-infected neonates present with cytomegalic inclusion disease, including growth retardation, hepatitis, and jaundice, along with brain development disorders in the form of microcephaly, encephalitis, seizures, and focal neurological signs. Approximately 10%–15% of infected infants who are asymptomatic at birth develop sensorineural hearing loss (Landolfo et al., 2003; Noyola et al., 2010). The clear health risks of exposure to HCMV make understanding the pathogenesis mechanism of this virus imperative.

The HCMV pathogenesis mechanisms center on two aspects: cell permissiveness for HCMV infection and the relationship between HCMV and the host cells (Landolfo et al., 2003). Based on viral protein synthesis and viral particle release, cell permissiveness for HCMV infection is divided into three categories: permissive, semi-permissive, and non-permissive. In permissive cells, the HCMV genome is temporally expressed. HCMV immediate-early (IE) proteins are synthesized immediately after virus entry into cells and activate the expression of early (E) genes and late (L) genes. These events result in the production of large amounts of viral particles and lysis of infected cells. In semi-permissive cells, the kinetics of HCMV protein expression is delayed, and very few viral progeny are produced. In non-permissive cells, viral proteins are not synthesized, and no subsequent viral progeny are produced.

Evidence has shown that epithelial cells, endothelial cells, and fibroblasts are susceptible to HCMV infection (Sinzger and Jahn, 1996). Our previous study also revealed that neural progenitor cells (NPCs) and their derived neuronal and glial cells were fully permissive for HCMV infection (Luo et al., 2008; Pan et al., 2013b). The monocyte/macrophage lineage in the peripheral blood has been shown to harbor infectious HCMV (Goodrum et al., 2002), and transfusion-mediated HCMV infection can be prevented by removal of the blood leucocytes (Musiani et al., 1984; Bhumbra et al., 1988; Gilbert et al., 1989). However, although HCMV viral transcripts and proteins have been observed in CD34<sup>+</sup> hematopoietic stem cells and CD14<sup>+</sup> monocytes, viral protein expression is delayed, and very few viral progeny are produced; thus, both cells are semi-permissive for HCMV infection. HCMV can also infect many other types of cells, and the virus has been detected in many organs that are mainly composed of parenchymal and mesenchymal cells and in which MSCs are primarily located. However, whether MSCs support HCMV replication and the infection status of HCMV in MSCs remain unclear.

To address this issue, MSCs were isolated from Wharton's jelly (WJ) of the human umbilical cord and identi-

fied by their plastic adherence, surface marker pattern, and differentiation capacity. Then, MSCs were infected with HCMV, and the infection status was characterized by assessing viral entry, replication, viral protein expression, and viral particle release using immunofluorescent (IFA), western blotting, and viral titer assays. The results indicated that MSCs were permissive for HCMV infection. The present study demonstrated that MSCs are a novel susceptible cell type for HCMV study and might prove useful to elucidate the pathogenesis mechanism of HCMV infection in non-nervous system diseases.

## MATERIALS AND METHODS

### Isolation and culture of MSCs

MSCs were isolated from WJ of the human umbilical cord using the tissue explant method (Han et al., 2013). In detail, 10–15-cm segments of umbilical cord tissue were obtained with informed consent from healthy full-term neonates after cesarean section at Zhongnan Hospital of Wuhan University. The segments were transferred to the laboratory in pre-cooled sterile Hank's balanced salt solution containing amphotericin B (5 µg/mL, Gibco), penicillin (200 units/mL, Gibco), and streptomycin (200 µg/mL, Gibco). The blood clots were washed off with ice-cold sterile phosphate buffered saline (PBS), and both ends were removed. The cord segment was then thoroughly washed with sterile PBS to remove the cord blood, dipped in 75% ethanol for 1 minute for sterilization, and washed with PBS twice to remove the residual ethanol. WJ was obtained after thoroughly peeling off the umbilical artery, veins, and amniotic membrane and was cut into 1-mm<sup>3</sup> pieces. These small pieces were evenly planted on a cell culture dish (Corning) and maintained on the bench at room temperature for 15 minutes to enhance sticking, and they were then incubated in complete culture medium at 37 °C in a 5% CO<sub>2</sub> atmosphere; the medium was changed every 3–4 days. When the MSCs released and attached to the culture surface about 2 weeks later, the pieces of WJ were carefully removed, and complete culture medium was added gently. The isolated MSCs were further cultured, the medium was refreshed every 2–3 days, and the cells were digested (0.25% trypsin-EDTA) and expanded in T75 flasks when at 80% confluence. The complete culture medium was Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) supplemented with 15% (vol/vol) fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL) (all from Life Technologies, California, USA).

### Differentiation of MSCs

To verify their multipotent differentiation potential,

MSCs were induced to differentiate to adipogenic (Adipo-) and osteogenic (Osteo-) cells as previously described with modifications (Jaiswal et al., 1997; Sibov et al., 2012). Briefly, early-passage MSCs (p4) were cultured in complete medium containing the corresponding stimulators for 21 days, fixed in 10% neutral buffered formalin for 60 minutes after a PBS rinse, and then stained with the corresponding dyes. Images were obtained with an inverted microscope (Nikon Eclipse TS100, Japan) equipped with a camera (Nikon CoolPix P6000, Japan). For adipogenic differentiation, 0.5  $\mu\text{mol/L}$  dexamethasone, 50  $\mu\text{mol/L}$  indomethacin, and 0.5 mmol/L isobutylmethylxanthine (IBMX) were applied as stimulators, and cells were stained with Oil Red O (all from Sigma). For osteogenic differentiation, the induction reagents were 2 mmol/L  $\beta$ -glycerophosphate, 0.1  $\mu\text{mol/L}$  dexamethasone, and 50  $\mu\text{mol/L}$  ascorbic acid, and the dye was Alizarin Red S (all from Sigma, USA). Fresh-cultured MSCs and 21-day cultured MSCs without exposure to stimulators were applied as controls.

### Virus infection

The HCMV Towne strain was propagated in human embryonic lung fibroblasts (HELs) as previously described (Pan et al., 2013a). Cells were infected with virus at an MOI of 3. After a 3-hour absorption period, the medium was replaced with fresh medium after a brief rinse with PBS. A mock-infection was prepared with an equal volume of HEL culture supernatant mixed with 1% DMSO and used as a negative control.

### Western blotting

At the indicated time points, cells were collected with a scraper, rinsed with ice-cold PBS, pelleted by centrifugation at 4 °C, snap-frozen in liquid nitrogen, and stored at -80 °C until needed. Then, cell pellets were resuspended in RIPA lysis buffer (Luo et al., 2007). After 5 minutes of centrifugation at 12000 $\times$ g, the supernatant was collected, and the protein concentration was determined using a BCA protein assay kit (Beyotime, China). Equivalent amounts of cell lysate (30  $\mu\text{g}$ ) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membranes (Millipore, USA). After blocking in 10% skimmed milk, the membranes were incubated with primary antibodies against the indicated target proteins and corresponding secondary antibodies. Signals were visualized using West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, USA), and images were obtained using a FluorChem HD2 System (Alpha Innotech, USA). The applied primary antibodies included mouse monoclonal antibodies (McAbs) against HCMV-IE1/2, HCMV-UL44, HCMV-pp65, HCMV-gB (Virsys, USA), and  $\beta$ -Actin (Santa Cruz, USA). Secondary

antibodies were horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (Amersham, Sweden).

### Immunofluorescence assay (IFA)

MSCs were seeded into 100-mm dishes ( $5 \times 10^5$  cells/dish) containing coverslips and cultured for 6 hours to let the cells equilibrate. At the indicated time points post-infection, the coverslips were collected, rinsed with PBS, and fixed with 4% formaldehyde. The immunofluorescence staining samples were processed as described previously (Duan et al., 2014). The following primary antibodies were used to analyze cell surface markers: mouse McAbs against CD73 (IgG<sub>3</sub>), CD44 (IgG<sub>1</sub>), CD105 (IgG<sub>2b</sub>), CD34 (IgG<sub>1</sub>), and HLA-DR (IgG<sub>1</sub>, Santa Cruz Biotechnology); mouse McAbs against HCMV-IE1 (IgG<sub>2a</sub>), HCMV-UL44 (IgG<sub>1</sub>), and HCMV-pp65 (IgG<sub>1</sub>, Virsys) were used for viral protein detection. Secondary antibodies included tetraethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgG<sub>3</sub> (SouthernBiotech, USA) and Alexa Fluor 488-conjugated goat anti-mouse IgG<sub>1</sub> (Invitrogen, USA). Hoechst dye 33342 (Thermo Fisher Scientific, USA) was applied to counterstain the cell nuclei. Images were taken with a Nikon Eclipse 80i fluorescence microscope equipped with a Nikon DS-Ri1 camera and NIS-Elements F3.0 software. For quantification analysis, at least 300 cells from a minimum five random fields were counted. The positive ratio was calculated as the percentage of cells expressing the corresponding viral protein in all cells stained by Hoechst.

### Plaque-forming assay

Cells were infected with HCMV at an MOI of 3. Aliquots of the supernatants were collected at the indicated time points to assess virus shedding. Supernatants were stored at -80 °C in 1% DMSO until used. Virus titer was determined by counting plaque-forming units (PFU) on HELs (Luo and Fortunato, 2007). Briefly, 200  $\mu\text{L}$  of a 10-fold serial dilution of viral supernatant was used to infect HELs. After attachment for 3 hours, cell growth medium containing 1% agarose was added to the inoculum. Plaques were counted at days 7 to 10 post-plating, with multiple wells seeded for each dilution, and an average titer was derived from the repeats.

### Statistical analyses

All images shown for the IFA and western blotting analyses are representative results from three independent experiments. Data for virus shedding and positive cell ratio quantification are presented as the mean  $\pm$  standard deviation (SD) from three independent experiments. A difference was considered to be statistically significant when  $P < 0.05$  with Student's *t*-tests.

## RESULTS

### Isolation and characterization of MSCs

Primary MSCs were isolated from WJ of the human umbilical cord and cultured as described in the Materials

and methods. According to the criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006), the plastic-adherence of MSCs was first examined. After 7 days, small, rod-like, and ir-

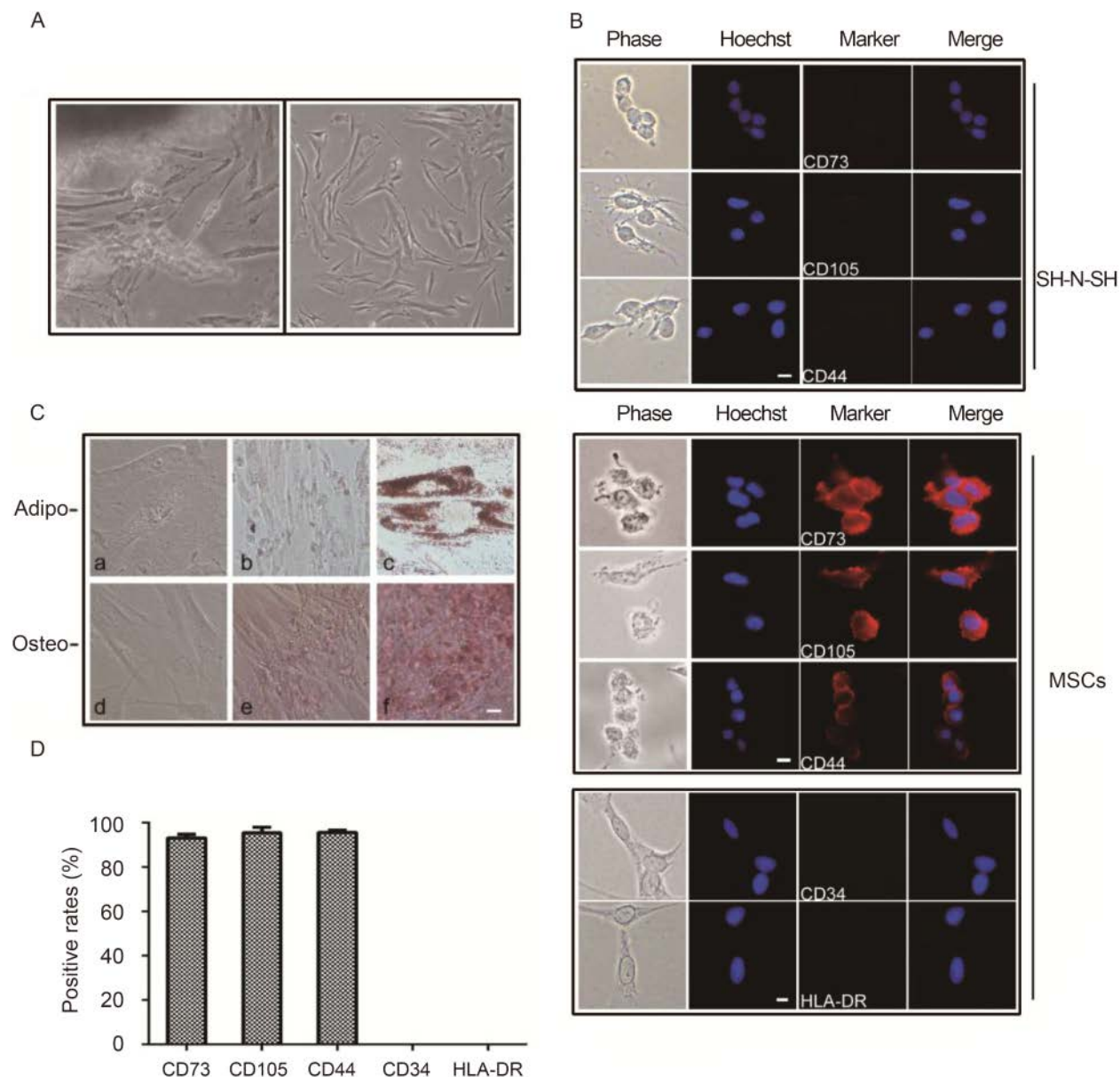


Figure 1. Isolation and characterization of human WJ-MSCs. (A) Morphology of primary cultured MSCs. Primary MSCs isolated from WJ of the human umbilical cord were cultured on a fibronectin-coated surface (left), and MSC sub-cultures were transferred from the primary culture to an uncoated surface (right). A typical plastic adherent fibroblast-like morphology is shown. (B) Identification of WJ-MSC surface markers. The expression levels of CD73, CD105, CD44, CD34, and HLA-DR in MSCs were examined by IFA. SH-N-SH cells were used as negative controls. Scale bar, 10  $\mu$ m. (C) Adipogenic and osteogenic differentiation of MSCs. Fresh-cultured MSCs (a, d) and 21-day cultured MSCs with (c, f) and without (b, e) induction were stained with Oil Red or Alizarin Red for adipogenic (Adipo-) and osteogenic (Osteo-) cells, respectively. Scale bar, 10  $\mu$ m. (D) The IFA images were used to calculate of frequencies of MSCs expressing the indicated surface markers. The data shown are the mean  $\pm$  SD from three independent experiments.

regularly shaped cells grew out of the explant, stuck to the culture dish, and formed colony forming units-fibroblasts (CFU-F) under the explant (data not shown). After 20 days, the primary cultured cells exhibited a fibroblast-like shape and grew around the explant with increased intercellular space (Figure 1A, left). Then, tissue explants were removed, and the culture medium was changed. After reaching 80%–90% confluence, the cells were trypsinized and transferred to a new culture surface. The sub-culture consisted of a mixture of mainly spindle-like or polygonal fibroblast-like shaped cells with a few irregularly shaped cells (Figure 1A, right), which fit the typical morphological description of MSCs.

To further characterize the isolated MSCs, we assessed surface markers, including CD105, CD73, CD44, CD34, and HLA-DR, using IFA. As shown in Figure 1B, the fibroblast-like shaped cells expressed CD73, CD105, and CD44, with positive ratios of 93.13%, 95.4%, and 95.63%, respectively (Figure 1D), while the negative control SH-N-SH cells expressed none of these markers. Neither CD34 nor HLA-DR expression was detected in the MSCs (Figure 1B).

Then, the multipotent differentiation potential, including adipogenesis (Figure 1C, a–c) and osteogenesis (Figure 1C, d–f), was assessed using different inducers to examine the differentiation ability of the isolated cells. After 21 days of induction with the corresponding reagents, cells were stained with Oil Red O or Alizarin Red S to visualize adipogenesis or osteogenesis, respectively. After adipo-induction, small red-stained oil drops were clearly observed in the cytoplasm (Figure 1C, c), and after osteo-induction, cells showed obvious red-stained calcium nodes (Figure 1C, f). As controls, fresh-cultured cells and 21-day cultured cells without induction were processed and stained in parallel. Fresh-cultured cells were not stained by either dye (Figure 1C, a and d), and 21-day cultured cells without induction showed only slight staining (Figure 1C, b and e).

All of the characteristics regarding morphology, surface marker expression pattern, and multipotent differentiation potential met the minimal criteria for defining MSCs recommended by the ISCT. Thus, these results confirmed that the cells isolated from WJ were MSCs.

### HCMV infection properties in MSCs

In permissive cells, the HCMV genome is temporally expressed. The IE genes are transcribed after virus entry and rely mainly on host cell cycle progression factors for expression. Prior to viral DNA synthesis, the early genes are expressed with the aid of IE gene products. Finally, L genes are transcribed after the initiation of viral DNA replication, with substantial release of virions. The morphology of infected cells gradually changes during the progression of virus infection, including vesicle forma-

tion, cell enlargement, and cell lysis (Lecointe et al., 1999). To reveal the permissiveness of MSCs for HCMV infection, the isolated MSCs were infected with HCMV at an MOI of 3, and the cells were observed at different time points post-infection. Upon HCMV infection, MSCs displayed visible morphological changes beginning at 24 hours post-infection (hpi) and exhibited an increasingly rounded-up shape from 48 hpi to 96 hpi (Figure 2). These results indicated the potential permissiveness of MSCs for HCMV infection.

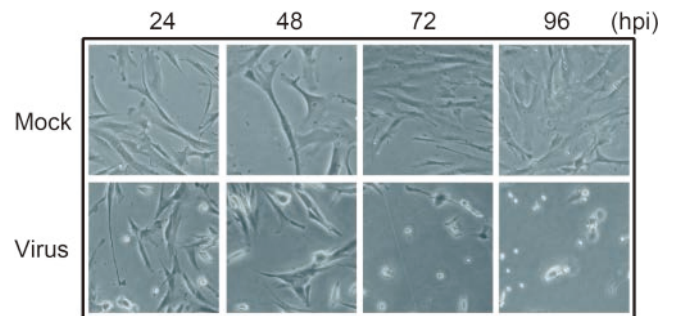


Figure 2. HCMV infection of MSCs. MSCs were infected with HCMV at an MOI of 3. Cytopathogenic effects of HCMV infection on MSCs were observed through an inverted microscope. Morphology of the cells at the indicated time points after HCMV or mock infection. Magnification,  $\times 100$ .

The viral life cycle includes viral entry, viral replication, protein expression, and viral progeny release. To further characterize the HCMV infection properties in MSCs, viral entry, replication initiation, and gene expression were examined using IFA and western blotting, and viral release was determined by PFU counting.

### Virus entry

After viral entry into permissive cells, some tegument proteins (such as pp65 and pp71) are also delivered into the cells and further transported into the nucleus. Thus, pp65 was chosen as an indicator to determine whether HCMV entry occurred in the first 24 hpi. As shown in Figure 3, pp65-positive cells were present as early as 4 hpi (Figure 3A), and the positive rate was  $94.33 \pm 8.02\%$  at 4 hpi and reached 100% after 8 hpi (Figure 3B).

### Initiation of viral replication

Entering the host cell is just the first step of the HCMV infection process, and it does not necessarily lead to the initiation of viral replication. Because the critical transactivating protein immediate early protein I (IE1) is one of the first strongly expressed proteins during HCMV replication, IE1 synthesis was measured as an indicator of viral replication initiation (Landolfo et al., 2003). As

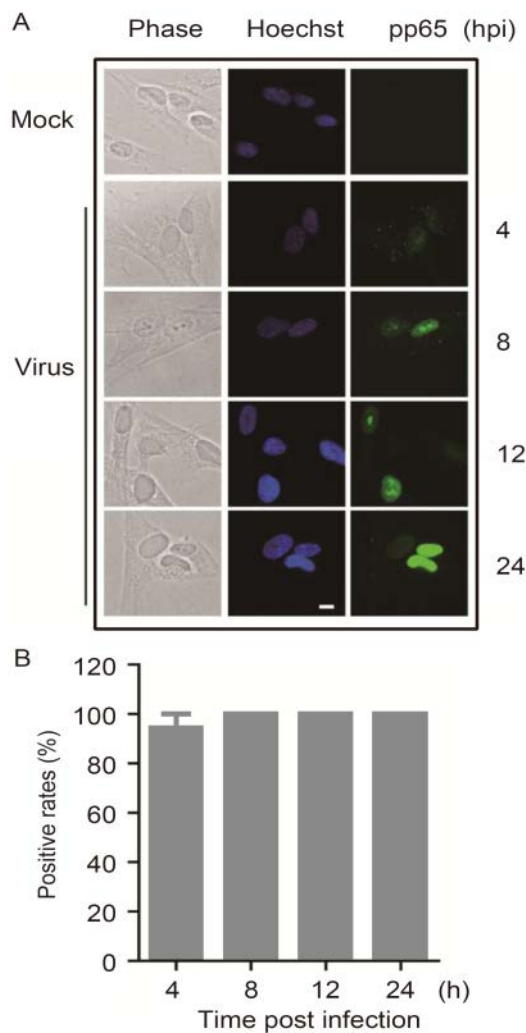


Figure 3. HCMV entry in MSCs. MSCs were infected with HCMV at an MOI of 3, and viral tegument protein pp65, serving as a virus entry indicator, was measured by IFA at the indicated time points (upper, scale bar, 10  $\mu$ m). The IFA images were used to calculate the percentage of pp65<sup>+</sup> cells, and the results are presented as the mean  $\pm$  SD of five independent experiments.

early as 4 hpi, IE1 was clearly observed in the nuclei of MSCs (Figure 4A), indicating that HCMV replication was initiated in these cells. The amount of IE1 continued to accumulate in the cells (Figure 4A), and the ratio of IE1-positive MSCs increased from  $5.44 \pm 1.53\%$  at 4 hpi to  $45.03 \pm 4.89\%$  at 36 hpi (Figure 4B).

**Viral genome replication**

The viral UL44 HCMV DNA polymerase processivity factor is a component of the viral replication center and is necessary for effective viral DNA replication. Thus, UL44 was measured by IFA to assess viral genome rep-

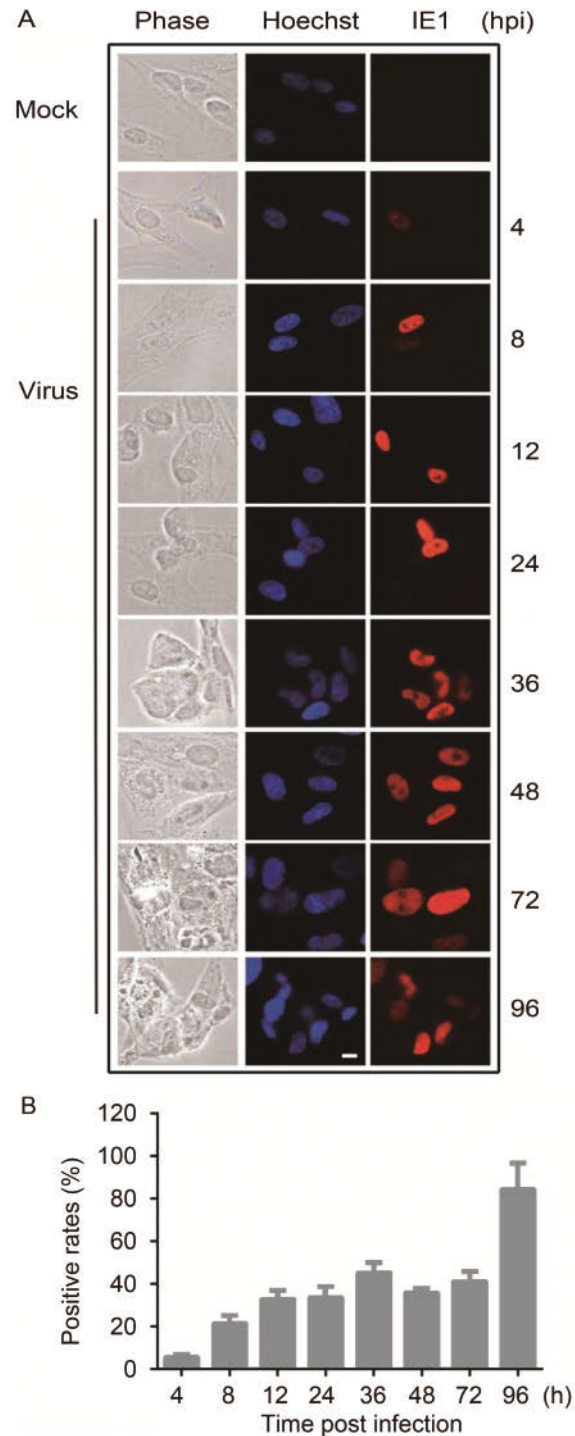


Figure 4. Initiation of HCMV replication in MSCs. MSCs were infected with HCMV at an MOI of 3, and IE1 expression, as an indicator of viral replication initiation, was examined by IFA at the indicated time points (upper, scale bar, 10  $\mu$ m). IE1<sup>+</sup> cells were counted according to the IFA data, and the frequency of IE1<sup>+</sup> cells is presented as the mean  $\pm$  SD from five independent experiments (lower).

lication. Cells containing UL44 were further categorized according to the subcellular location as UL44<sup>+</sup> cells and UL44 foci<sup>+</sup> cells. The designation of UL44<sup>+</sup> indicated that positive signals existed in the cells. The designation of UL44 foci<sup>+</sup> indicated that positive signals existed in the nuclei, formed foci, and then gathered gradually starting at 24 hpi, which represented the hallmark of HCMV genome replication (Luo et al., 2010). In Figure 5A, UL44<sup>+</sup> cells and UL44 foci<sup>+</sup> cells are indicated by arrows and triangles, respectively. At 12 hpi, the UL44<sup>+</sup> signals exhibited diffuse staining in MSCs. After 24 hpi, some cells had areas of accumulated signals underlying a diffuse pattern and were designated UL44 foci<sup>+</sup> cells, indicating viral replication sites. In addition, as previously demonstrated by a number of groups (Penfold and Mocarski, 1997; Luo et al., 2008), the accumulation of UL44 signal increased after 36 hpi, mainly forming bipolar foci at 36 hpi and large nuclear domains at 48 hpi. To evaluate the replication efficiency of HCMV in MSCs, the UL44 foci<sup>+</sup>/IE1<sup>+</sup> values were calculated at different time points. The results showed that the value UL44 foci<sup>+</sup>/IE1<sup>+</sup> values sharply increased after 36 hpi, from  $20.87 \pm 3.32\%$  at 24 hpi to  $76.22 \pm 4.08\%$  at 36 hpi and to  $92.04 \pm 8.72\%$  at 96 hpi (Figure 5B), indicating a high level of viral genome replication after 24 hpi in MSCs. Together, the results indicate that efficient replication of the viral genome began shortly after viral entry into MSCs.

### Viral gene expression

After the initiation of viral replication by IE gene products,

the viral E and L gene products are synthesized in a time-dependent manner. During the time course of viral infection, the major immediate early proteins IE1 and IE2 could be detected at 4 hpi. The early proteins UL44 and pp65 with different subtypes were present at 48 hpi. The representative late stage protein gB was also detected at 48 hpi (Figure 6A).

### Virus progeny release

After viral replication and viral protein expression, viral progeny products are assembled and released. To further analyze viral progeny, supernatants of HCMV-infected MSCs were collected at designated times to determine viral titer by PFU counting. As shown in Figure 6B, the viral titer in MSCs presented a typical logarithmic growth pattern and progressed into a slow growth state (Figure 6B). These results further indicated that MSCs are fully permissive for HCMV infection.

Thus, the following results indicate that MSCs are fully permissive for HCMV infection: viral entry into MSCs as early as 4 hpi, initiation of viral replication at 4 hpi, and release of viral progeny products.

### DISCUSSION

MSCs have great therapeutic potential due to their capacity for self-renewal and multi-potency, and they are capable of differentiating into bone, cartilage, fat, tendon, muscle, and marrow stroma (Pittenger et al., 1999). HCMV is a ubiquitous pathogen that can infect

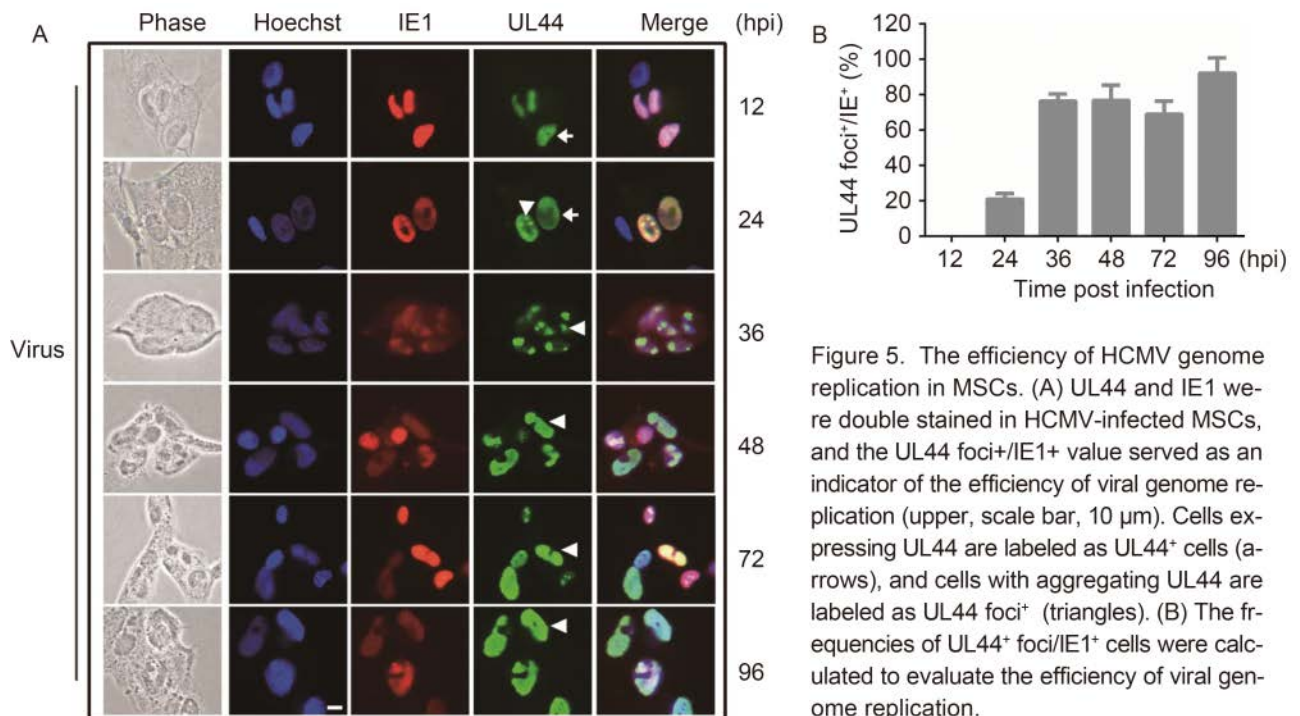


Figure 5. The efficiency of HCMV genome replication in MSCs. (A) UL44 and IE1 were double stained in HCMV-infected MSCs, and the UL44 foci/IE1<sup>+</sup> value served as an indicator of the efficiency of viral genome replication (upper, scale bar, 10 μm). Cells expressing UL44 are labeled as UL44<sup>+</sup> cells (arrows), and cells with aggregating UL44 are labeled as UL44 foci<sup>+</sup> (triangles). (B) The frequencies of UL44<sup>+</sup> foci/IE1<sup>+</sup> cells were calculated to evaluate the efficiency of viral genome replication.

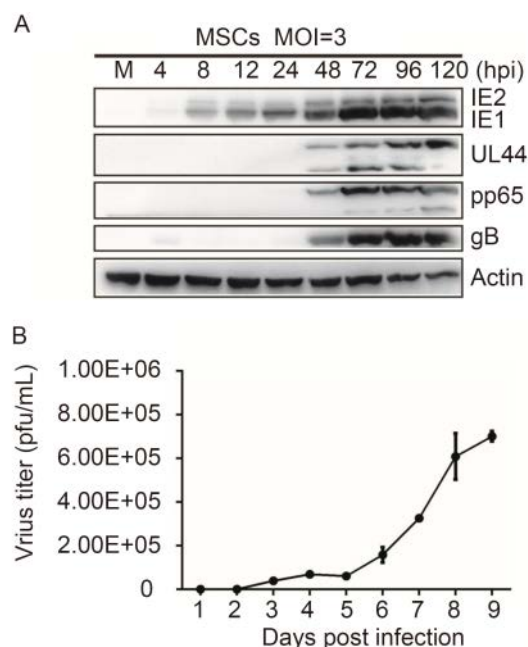


Figure 6. Viral protein expression and viral particle shedding. (A) Representative viral protein expression in HCMV-infected MSCs. Cells were collected at the indicated time points, and western blotting was performed to examine the expression of viral proteins.  $\beta$ -actin served as a control for cell amount normalization. (B) MSCs were infected with HCMV (Towne strain) at an MOI of 3. Supernatants were collected at the indicated time points for virus shedding titration.

most organs, including the brain, lungs, heart, salivary gland, liver, kidney, and bone marrow. MSCs are involved in multi-organ formation, but whether MSCs play a role in the pathogenesis of congenital HCMV infection remains unclear. In this study, we determined the susceptibility of MSCs for HCMV infection and confirmed that MSCs are fully permissive for HCMV infection. These results represent new information that will aid in determining the pathogenesis mechanism of HCMV infection.

A large number of reports have shown that MSCs can be derived from various tissues, including bone marrow (BM), periosteum, adipose tissue, skeletal muscle, deciduous teeth, fetal pancreas, lung, liver, cord blood, and umbilical cord tissues (Zuk et al., 2001; Lu et al., 2006; Wei et al., 2013; Rylova et al., 2015). Previously, BM was the major source of MSCs for use in cell therapy. However, the aspiration of BM involves invasive procedures, and the frequency and differentiation potential of BM-MSCs decrease significantly with age. Cord blood and umbilical cord tissue might be ideal sources of MSCs because of their advantages of having very similar cell characteristics, being widely available, requiring a non-

invasive collection procedure, and being associated with relatively low ethical risks. Regarding the human umbilical cord, MSCs can be found in the umbilical cord lining, sub-endothelial layer, perivascular zone, and WJ (Watson et al., 2015). Among the regions of the umbilical cord, WJ is the best source of cells because MSCs in WJ are maintained in an early embryologic phase and therefore have retained some of their primitive stemness properties (Watson et al., 2015). Therefore, we chose WJ as the MSC source in this study. Following the criteria for the identification of MSCs proposed by the ISCT, we examined the plastic adherence, surface markers, and differentiation properties of the isolated MSCs. The results indicated that the cells isolated from WJ were MSCs because they met all criteria.

The results of the cell susceptibility analysis showed that MSCs are fully permissive for HCMV infection, as indicated by viral pp65 protein entry into cells immediately after HCMV infection, viral IE protein expression, viral replication, viral E and L protein expression, and viral particle release. MSCs are found in almost all tissues and are a subset of non-hematopoietic stem cells that originate from the mesoderm during embryonic development. MSCs can differentiate into not only mesoderm lineages but also ectodermic and endodermic cells (Bruder et al., 1994; Wei et al., 2013). Thus, if MSCs were infected with HCMV in an embryogenesis stage, the transduction of a series of signals might be affected by the infection and lead to abnormal morphogenesis and organ functions, further exacerbating the disease in HCMV-infected neonates.

Because of their multi-lineage differentiation potential and immunomodulatory functions, MSCs have promise for use in the treatment of numerous diseases. A large number of studies have assessed the therapeutic efficacy of MSCs on disease progression in experimental animal models and human clinical trials and found that MSC administration can promote tissue repair by secreting soluble factors that alter the tissue microenvironment or inducing cells to transdifferentiate into epithelial cells and similar lineages (Phinney and Prockop, 2007). However, HCMV infection might influence the effective modulation or transdifferentiation of MSCs and potentially lead to the disordered repair of tissues. In addition, infection of MSCs with HCMV possibly leads to complications such as pneumonia, myocarditis, retinitis, and gastrointestinal ulceration. HCMV infection also leads to disease in various tissues, including the brain, lung, liver, and gastrointestinal tract (Landolfo et al., 2003).

MSCs are located within the connective tissue compartments of many organs and organ systems and thus have a widespread distribution throughout the body (Young et al., 1995). In addition, the therapeutic mechanisms of MSCs include many cytokines, transcriptional factors, and signaling pathways (Mrugala et al., 2009; Le

Blanc and Mougiakakos, 2012; Dudakovic et al., 2015; Laranjeira et al., 2015; Sela et al., 2015; Tan et al., 2015). Among the various factors involved, HCMV infection can activate transforming growth factor- $\beta$  in renal tubular epithelial cells (Shimamura et al., 2010) and osteosarcoma cell lines (Kwon et al., 2004), down-regulate epidermal growth factor receptor (EGFR) in human fetal lung fibroblasts (Beutler et al., 2003), induce platelet-derived growth factor receptor (PDGFR) expression in human vascular smooth muscle cells (Reinhardt et al., 2005a), and dysregulate PDGFR, EGFR, Wnt/ $\beta$ -catenin, Notch, and NF- $\kappa$ B signaling pathways (Cinatl et al., 2001; Fairley et al., 2002; Reinhardt et al., 2005b; Bentz and Yurochko, 2008; Isern et al., 2011; Angelova et al., 2012; El-Shinawi et al., 2013; Kapoor et al., 2013; Mathers et al., 2014; Li et al., 2015). The exact nature of the relationship between MSCs and the pathogenesis of HCMV infection remains to be determined.

In conclusion, MSCs are fully permissive for HCMV infection, as indicated by viral entry into MSCs as early as 4 hpi, replication initiation, viral protein expression, and viral progeny product release. The results of this study provide a new perspective on the pathogenesis of HCMV infection.

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## COMPLIANCE WITH ETHICS GUIDELINES

There were no conflicts of interest in the study. Additional informed consent was obtained from all patients for which identifying information is included in this article.

## AUTHOR CONTRIBUTIONS

GHQ conceived and performed the experiments. MHL and SC designed and oversaw the study. FZ contributed to the western blot and IFA analyses.

## REFERENCES

- Aggarwal S, Pittenger MF. 2005. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*, 105: 1815–1822.
- Angelova M, Zvezdaryk K, Ferris M, Shan B, Morris CA, Sullivan DE. 2012. Human cytomegalovirus infection dysregulates the canonical Wnt/ $\beta$ -catenin signaling pathway. *PLoS Pathog*, 8: e1002959.
- Bale JF, Jr. 1984. Human cytomegalovirus infection and disorders of the nervous system. *Arch Neurol*, 41: 310–320.
- Bentz GL, Yurochko AD. 2008. Human CMV infection of endothelial cells induces an angiogenic response through viral binding to EGF receptor and  $\beta$ (1) and  $\beta$ (3) integrins. *Proceedings of the National Academy of Sciences of the United States of America*, 105: 5531–5536.
- Beutler T, Hoflich C, Stevens PA, Kruger DH, Prosch S. 2003. Downregulation of the epidermal growth factor receptor by human cytomegalovirus infection in human fetal lung fibroblasts. *Am J Respir Cell Mol Biol*, 28: 86–94.
- Bhumbra NA, Lewandowski P, Lau P, Sererat M, Satish M, Nankervis GA. 1988. Evaluation of a prescreening blood donor program for prevention of perinatal transfusion-acquired cytomegalovirus (CMV) infection. *J Perinat Med*, 16: 127–131.
- Bruder SP, Fink DJ, Caplan AI. 1994. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem*, 56: 283–294.
- Cinatl J, Margraf S, Vogel JU, Scholz M, Cinatl J, Doerr HW. 2001. Human cytomegalovirus circumvents NF- $\kappa$ B dependence in retinal pigment epithelial cells. *J Immunol*, 167: 4771–4771.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, 8: 315–317.
- Duan YL, Ye HQ, Zavala AG, Yang CQ, Miao LF, Fu BS, Seo KS, Davrinche C, Luo MH, Fortunato EA. 2014. Maintenance of large numbers of virus genomes in human cytomegalovirus-infected T98G glioblastoma cells. *J Virol*, 88: 3861–3873.
- Dudakovic A, Camilleri ET, Lewallen EA, McGee-Lawrence ME, Riester SM, Kakar S, Montecino M, Stein GS, Ryoo HM, Dietz AB, Westendorf JJ, van Wijnen AJ. 2015. Histone deacetylase inhibition destabilizes the multi-potent state of uncommitted adipose-derived mesenchymal stromal cells. *J Cell Physiol*, 230: 52–62.
- El-Shinawi M, Mohamed HT, El-Ghonaimy EA, Tantawy M, Younis A, Schneider RJ, Mohamed MM. 2013. Human cytomegalovirus infection enhances NF- $\kappa$ B/p65 signaling in inflammatory breast cancer patients. *PLoS One*, 8: e55755.
- Fairley JA, Baillie J, Bain M, Sinclair JH. 2002. Human cytomegalovirus infection inhibits epidermal growth factor (EGF) signalling by targeting EGF receptors. *J Gen Virol*, 83: 2803–2810.
- Gilbert GL, Hayes K, Hudson IL, James J. 1989. Prevention of transfusion-acquired cytomegalovirus infection in infants by blood filtration to remove leucocytes. Neonatal Cytomegalovirus Infection Study Group. *Lancet*, 1: 1228–1231.
- Goodrum FD, Jordan CT, High K, Shenk T. 2002. Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency. *Proc Natl Acad Sci U S A*, 99: 16255–16260.
- Han YF, Tao R, Sun TJ, Chai JK, Xu G, Liu J. 2013. Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods. *Cytotechnology*, 65: 819–827.
- Isern E, Gustems M, Messerle M, Borst E, Ghazal P, Angulo A. 2011. The Activator Protein 1 Binding Motifs within the Human Cytomegalovirus Major Immediate-Early Enhancer Are Functionally Redundant and Act in a Cooperative Manner with the NF- $\kappa$ B Sites during Acute Infection. *J Virol*, 85: 1732–1746.
- Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. 1997. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J Cell Biochem*, 64: 295–312.
- Kapoor A, He R, Venkatadri R, Forman M, Arav-Boger R. 2013. Wnt modulating agents inhibit human cytomegalovirus replication. *Antimicrob Agents Ch*, 57: 2761–2767.
- Kwon YJ, Kim DJ, Kim JH, Park CG, Cha CY, Hwang ES. 2004. Human cytomegalovirus (HCMV) infection in osteosarcoma

- cell line suppresses GM-CSF production by induction of TGF- $\beta$ . *Microbiol Immunol*, 48: 195–199.
- Landolfo S, Gariglio M, Gribaudo G, Lembo D. 2003. The human cytomegalovirus. *Pharmacol Ther*, 98: 269–297.
- Laranjeira P, Pedrosa M, Pedreiro S, Gomes J, Martinho A, Antunes B, Ribeiro T, Santos F, Trindade H, Paiva A. 2015. Effect of human bone marrow mesenchymal stromal cells on cytokine production by peripheral blood naive, memory, and effector T cells. *Stem Cell Res Ther*, 6: 3.
- Le Blanc K, Mougiakakos D. 2012. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol*, 12: 383–396.
- Lecointe D, Hery C, Janabi N, Dussaix E, Tardieu M. 1999. Differences in kinetics of human cytomegalovirus cell-free viral release after in vitro infection of human microglial cells, astrocytes and monocyte-derived macrophages. *J Neurovirol*, 5: 308–313.
- Li XJ, Liu XJ, Yang B, Fu YR, Zhao F, Shen ZZ, Miao LF, Rayner S, Chavanas S, Zhu H, Britt WJ, Tang Q, McVoy MA, Luo MH. 2015. Human Cytomegalovirus Infection Dysregulates the Localization and Stability of NICD1 and Jag1 in Neural Progenitor Cells. *J Virol*, 89: 6792–6804.
- Lu LL, Liu YJ, Yang SG, Zhao QJ, Wang X, Gong W, Han ZB, Xu ZS, Lu YX, Liu D, Chen ZZ, Han ZC. 2006. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica*, 91: 1017–1026.
- Luo MH, Fortunato EA. 2007. Long-term infection and shedding of human cytomegalovirus in T98G glioblastoma cells. *J Virol*, 81: 10424–10436.
- Luo MH, Hannemann H, Kulkarni AS, Schwartz PH, O'Dowd JM, Fortunato EA. 2010. Human cytomegalovirus infection causes premature and abnormal differentiation of human neural progenitor cells. *J Virol*, 84: 3528.
- Luo MH, Rosenke K, Czornak K, Fortunato EA. 2007. Human cytomegalovirus disrupts both ataxia telangiectasia mutated protein (ATM)-and ATM-Rad3-related kinase-mediated DNA damage responses during lytic infection. *J virol*, 81: 1934.
- Luo MH, Schwartz PH, Fortunato EA. 2008. Neonatal neural progenitor cells and their neuronal and glial cell derivatives are fully permissive for human cytomegalovirus infection. *J Virol*, 82: 9994–10007.
- Mathers C, Schafer X, Martinez-Sobrido L, Munger J. 2014. The human cytomegalovirus UL26 protein antagonizes NF-kappaB activation. *J Virol*, 88: 14289–14300.
- Mrugala D, Dossat N, Ringe J, Delorme B, Coffy A, Bony C, Charbord P, Haupl T, Daures JP, Noel D, Jorgensen C. 2009. Gene Expression Profile of Multipotent Mesenchymal Stromal Cells: Identification of Pathways Common to TGF  $\beta$  3/BMP2-Induced Chondrogenesis. *Cloning and Stem Cells*, 11: 61–75.
- Musiani M, Zerbini M, Carpi C, Plazzi M, Sermasi G, Belletti D, Sacchi R. 1984. Serological screening for the prevention of transfusion-acquired cytomegalovirus infection. *J Infect*, 9: 148–152.
- Noyola DE, Jimenez-Capdeville ME, Demmler-Harrison GJ. 2010. Central nervous system disorders in infants with congenital cytomegalovirus infection. *Neurol Res*, 32: 278–284.
- Pan X, Li XJ, Liu XJ, Yuan H, Li JF, Duan YL, Ye HQ, Fu YR, Qiao GH, Wu CC, Yang B, Tian XH, Hu KH, Miao LF, Chen XL, Zheng J, Rayner S, Schwartz PH, Britt WJ, Xu J, Luo MH. 2013a. Later Passage Neural Progenitor Cells from Neonatal Brain Are More Permissive for Human Cytomegalovirus Infection. *J Virol*. 87: 10968–10979.
- Pan X, Li XJ, Liu XJ, Yuan H, Li JF, Duan YL, Ye HQ, Fu YR, Qiao GH, Wu CC, Yang B, Tian XH, Hu KH, Miao LF, Chen XL, Zheng J, Rayner S, Schwartz PH, Britt WJ, Xu J, Luo MH. 2013b. Later passages of neural progenitor cells from neonatal brain are more permissive for human cytomegalovirus infection. *J Virol*, 87: 10968–10979.
- Penfold ME, Mocarski ES. 1997. Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. *Virology*, 239: 46–61.
- Phinney DG, Prockop DJ. 2007. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells*, 25: 2896–2902.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science*, 284: 143–147.
- Pittenger MF, Martin BJ. 2004. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circulation Research*, 95: 9–20.
- Reinhardt B, Mertens T, Mayr-Beyrle U, Frank H, Lüske A, Schierling K, Waltenberger J. 2005a. HCMV infection of human vascular smooth muscle cells leads to enhanced expression of functionally intact PDGF  $\beta$ -receptor. *Cardiovascular Research*, 67: 151–160.
- Reinhardt B, Mertens T, Mayr-Beyrle U, Frank H, Luske A, Schierling K, Waltenberger J. 2005b. HCMV infection of human vascular smooth muscle cells leads to enhanced expression of functionally intact PDGF beta-receptor. *Cardiovasc Res*, 67: 151–160.
- Rylova YV, Milovanova NV, Gordeeva MN, Savilova AM. 2015. Characteristics of Multipotent Mesenchymal Stromal Cells from Human Terminal Placenta. *Bull Exp Biol Med*, 159: 253–257.
- Sela M, Tirza G, Ravid O, Volovitz I, Solodееv I, Friedman O, Zippori D, Gur E, Krelm Y, Shani N. 2015. NOX1-induced accumulation of reactive oxygen species in abdominal fat-derived mesenchymal stromal cells impinges on long-term proliferation. *Cell Death Dis*, 6: e1728.
- Shimamura M, Murphy-Ullrich JE, Britt WJ. 2010. Human cytomegalovirus induces TGF- $\beta$ 1 activation in renal tubular epithelial cells after epithelial-to-mesenchymal transition. *PLoS Pathog*, 6: e1001170.
- Sibov TT, Severino P, Marti LC, Pavon LF, Oliveira DM, Tobo PR, Campos AH, Paes AT, Amaro E, Jr., L FG, Moreira-Filho CA. 2012. Mesenchymal stem cells from umbilical cord blood: parameters for isolation, characterization and adipogenic differentiation. *Cytotechnology*, 64: 511–521.
- Sinzger C, Jahn G. 1996. Human cytomegalovirus cell tropism and pathogenesis. *Intervirology*, 39: 302–319.
- Tan SL, Ahmad TS, Ng WM, Azlina AA, Azhar MM, Selvaratnam L, Kamarul T. 2015. Identification of Pathways Mediating Growth Differentiation Factor5-Induced Tenogenic Differentiation in Human Bone Marrow Stromal Cells. *PLoS One*, 10: e0140869.
- Watson N, Divers R, Kedar R, Mehindru A, Borlongan MC, Borlongan CV. 2015. Discarded Wharton jelly of the human umbilical cord: a viable source for mesenchymal stromal cells. *Cytotherapy*, 17: 18–24.
- Wei X, Yang X, Han ZP, Qu FF, Shao L, Shi YF. 2013. Mesenchymal stem cells: a new trend for cell therapy. *Acta Pharmacol Sin*, 34: 747–754.
- Young HE, Mancini ML, Wright RP, Smith JC, Black AC, Reagan CR, Lucas PA. 1995. Mesenchymal Stem-Cells Reside within the Connective Tissues of Many Organs. *Developmental Dynamics*, 202: 137–144.
- Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*, 7: 211–228.

