The hUC-MSCs cell line CCRC-1 represents a novel, safe and high-yielding HDCs for the production of human viral vaccines

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MRC-5 represents the most frequent human diploid cells (HDCs)-type cell substrate in the production of human viral vaccines. However, early-passage MRC-5 is diminishing and, due to both technical and ethical issues, it is extremely difficult to derive novel HDCs from fetal lung tissues, which are the common sources of HDCs. Our previous studies suggested that human umbilical cord may represent an alternative but convenient source of new HDCs. Here, we established a three-tiered cell banking system of a hUC-MSC line, designated previously as Cell Collection and Research Center-1 (CCRC-1). The full characterization indicated that the banked CCRC-1 cells were free from adventitious agents and remained non-tumorigenic. The CCRC-1 cells sustained its rapid proliferation even at passage 30 and were susceptible to the infection of a wide spectrum of viruses. Interestingly, the CCRC-1 cells showed much higher production of EV71 or Rubella viruses than MRC-5 and Vero cells when growing in serum-free medium. More importantly, the EV71 vaccine produced from CCRC-1 cells induced immunogenicity while eliciting no detectable toxicities in the tested mice. Collectively, these studies further supported that CCRC-1, and likely other hUC-MSCs as well, may serve as novel, safe and high-yielding HDCs for the production of human viral vaccines.
propagation capacity as well as ethical issues, continuous supply of low-passage HDCs has always being a critical problem in the field of vaccine production.

Mesenchymal stem cells (MSCs) are a group of fibroblast-like cells with abilities to self-renew and to differentiate into multiple cell lineages, such as osteocytes, chondrocytes and adipocytes. A unique feature of MSCs in the focus of recent studies is its unique immunomodulatory activities, which have been implicated in the treatment or prevention of various inflammatory and autoimmune diseases. However, developing MSCs as novel cell substrates for the production of viral vaccines has rarely been explored.

Interestingly, our recent studies demonstrated that many HDCs established from fetal lungs, such as MRC-5, exhibited several critical properties of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs), including cell morphology, growth activity, expression of cell surface markers, abilities to differentiate into multiple cell lineages and immunomodulatory activities. In the meantime, it was found that the (Cell Collection and Research Center-1) cells, an hUC-MSC cell line reported in the previous studies, sustained primitive characteristics during extensive expansion and exhibited a similar sensitivity to the infection of EV71 and Rubella viruses as MRC-5, thus suggesting that hUC-MSCs may meet the same requirements as the traditional HDCs for the production of human vaccines.

In the present study, to further develop CCRC-1 as a novel HDC for the production of human vaccines, we first established a three-tiered banking system for CCRC-1, intensely characterized the banked cells for growth activities and tumorigenicity, and then evaluated the susceptibility of the cells to a wide spectrum of viruses and the growth and propagation of both EV71 and Rubella viruses in the cells. With a greater focus on EV71, we also compared the immunogenicity and safety of EV71 vaccines produced in CCRC-1 cells with that from MRC-5 and Vero cells. Finally, we demonstrated that different strains of hUC-MSCs exhibited a similar susceptibility to both EV71 and Rubella infections, therefore concluding that CCRC-1, and perhaps other hUC-MSC cell lines as well, may be used as novel HDCs for the production of human viral vaccines.

**Materials and Methods**

**Materials.** Cells. MRC-5, Vero and RK-13 cells were obtained from the American Type Culture Collection (Rockville, MD, USA), all hUC-MSCs were isolated from Wharton’s jelly of human umbilical cord and fully characterized. MRC-5 was cultured in MEM supplemented with 10% FBS (Gibco, Grand Island, NY, USA), Vero and RK-13 were cultured in MEM supplemented with 10% NBS (Sijiqing, Zhejiang, China), hUC-MSCs were cultured in α-MEM supplemented with 10% FBS.

Virus. EV71 strain 523-07T (EV71/523-07T), Sendai virus (SEV), Adenovirus type 5 (ADV-5) and Herpes simplex virus 2 (HSV-2) were all derived from our institute; EV71 vaccine strain SH06 (EV71/S06) and Rubella vaccine strain RA27/3 (RUV/RA27/3) were provided by Sinovac Biotech Ltd (Beijing, China); Measles vaccine strain Chang-47 (MV/Chang-47) and Varicella Zoster virus vaccine strain Oka (VZV/Oka) were from Changchun Institute of Biological Products (Changchun, China); Japanese encephalitis vaccine strain SA14-14-2 (JEV/SA14-14-2) was from Chengdu Institute of Biological Products (Chengdu, China).

Animals. Suckling mice, adult mice, guinea pigs, rabbits and female nude mice were purchased from the Laboratory Animal Center of National Institutes for Food and Drug Control (NIFDC) (Beijing, China). All studies involving animals were conducted in conformity with institutional guidelines concerning animal use and care and the relevant protocols approved by the NIFDC Institutional Animal Care and Use Committee were followed.

Generation and characterization of primary, master and working cell banks. The CCRC-1 cells at passage 5 following the initial isolation and proliferation were used as cell seeds, which were expanded in 175-cm² cell culture flasks through consecutive passages to generate a primary cell bank (PCB). Following an industry standard procedure for three-tiered cell banking, a master cell bank (MCB) and a working cell bank (WCB) were sequentially generated using the cells from the PCB. A portion of the cells from each bank were intensely evaluated for their freedom from contamination of mycoplasma, bacteria, fungi and viruses according to the recommendations of World Health Organization (WHO) Guidelines as well as the requirements from Chinese Pharmacopeia. Briefly, the presence of bacteria and fungi in CCRC-1 cells were tested using the cul-

Tumor formation assay. The tumorigenicity of CCRC-1 cells at different passage levels was evaluated by their abilities to form tumors in adult nude mice. Briefly, the nude mice were injected subcutaneously at the dorsal medial sites with 1×10⁷ cells suspended in 200μl serum-free medium. The injection with 1×10⁸ Hela cells suspended in 200μl of MEM basal medium served as positive control. During a 3-month observation period, the animals were examined for any abnormalities in appearance and behavior, and palpated twice a week to detect nodule formation at the site of inoculation. At the end of the experiment, the animals were euthanized and necropsied. The tissues of the skin at the injection site, and the organs of the adjacent lymph nodes, heart, liver, spleen, lungs, kidney, and any gross lesions were collected. They were then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, stained and finally examined under a microscope by a certified pathologist.
**hTERT activity assay.** The hTERT activity in CCRC-1 cells at different passage levels and in A549 human lung cancer cells was determined by using TRAPEze XL Telomerase Detection Kit (Millipore, Billerica, MA, USA) according to the procedures described in the manufacturer’s instruction. Briefly, PCR amplification was performed and the products were transferred to 96-well plates. The intensity of the fluorescent signal emitted by the PCR products was measured with Gemini XPS Microplate Reader (Molecular Devices, USA) and used to determine the hTERT activity."
cervical dislocation and the major tissues or organs of each mouse were biopsied and analyzed for any pathological findings.

**Statistical analysis.** Statistical analyses were performed using SPSS version 17.0. Results were presented as mean ± standard deviation (SD) of the data from three independent experiments. For statistical comparison between groups, Student’s t test was used, with a p value less than 0.05 considered significant.

**Results**

**Generation and characterization of CCRC-1 cell banks.** Following our previous study, to further validate that hUC-MSCs can potentially serve as a novel group of HDCs, and to establish CCRC-1 as a novel HDC cell substrate for vaccine production, the CCRC-1 cells cultured in α-MEM complete medium was continuously expanded by observing an industry cell banking standard to generate a three-tiered cell banking system, in which the PCB was at passage 9, the MCB at passage 13, and the WCB at passage 171–4. By following the requirements of the relevant bank characterization guidelines18,19, the cell banks were fully characterized for their freedom from contamination with a spectrum of adventitious agents. The bank characterization was performed by utilizing both in vitro and in vivo tests, including the inoculation of the testing materials into indicator cells, specific pathogen free (SPF) embryonated eggs, mice, guinea pigs, or rabbits (Table 1). As summarized in Table 1, the three-tiered banks showed freedom from contamination by bacteria, fungus, mycoplasmas, species-specific viruses, retroviruses or any unspecified adventitious agents.

**CCRC-1 cells maintain non-tumorigenic feature and a high growth activity during the extended proliferation.** To meet the requirements of non-tumorigenicity for HDCs2, the CCRC-1 cells at various passage levels were evaluated using nude mice tumor formation assay. As a result, while the Hela cells, used as a positive control in the assay, readily showed a progressive tumor formation starting at the first 30 day after inoculation in all animals tested, no palpable nodules could be detected in the mice inoculated with CCRC-1 cells at passage levels of P5, P15 and P30 during the entire observation period. In addition, the biopsied tissues showed no existence of tumor cells under microscopic examination in all test groups (Table 2).

To further exclude the tumorigenic transformation of CCRC-1 cells, we also evaluated the hTERT activity of the cells at passage levels of 10, 15, 20, 25 and 30. The results showed that, while the control A549 lung cancer cells exhibited approximately 1200 TPG of hTERT activity, the CCRC-1 cells at all passages exhibited an extremely low hTERT activity (Fig. 1).

To determine whether the extended proliferation of CCRC-1 could reduce its growth activity, the cells at different passage levels were tested for their growth pattern and doubling time, the parameters commonly used to indicate the proliferation potential of HDCs5. As shown in Fig. 2, the CCRC-1 cells at different passage levels during continuous growth for 8 days did not show a significant difference in both cell morphology and proliferation rate. In addition, it was observed that the peak cell densities of CCRC-1 and MRC-5 cells at passage 30 were (1.08 ± 0.16) × 10^5 cells/cm² and (0.86 ± 0.10) × 10^5 cells/cm² (Fig. 2), respectively, thus suggesting that the CCRC-1 cells could reach an even higher peak density than MRC-5 cells at the same passage level (P < 0.05). By

| Test Entities                                      | Methods                                                                 | Result   |
|----------------------------------------------------|-------------------------------------------------------------------------|----------|
| Bacterial and fungi                                | Sterility test*                                                         | Negative |
| Mycoplasmas                                        | Culture method*                                                         | Negative |
| Indicator cell inoculation and staining*            |                                                                         | Negative |
| Viruses                                            | In vitro tests using indicator cells*                                   | Negative |
|                                                     | Nucleic acid testing for species-specific viruses                       | Negative |
|                                                     | In vivo animal inoculation tests using the embryonated eggs, suckling mice, adult mice and rabbit and guinea pigs | Negative |
|                                                     | Transmission Electron Microscopy test for viral particles               | Negative |
|                                                     | Tests for retroviruses*                                                 | Negative |

Table 1. The CCRC-1 cell bank characterization for testing adventitious agents. In the cell bank characterization, the MCB (master cell bank) was comprehensively tested for all testing items by all testing methods, whereas the PCB (primary cell bank) and WCB (working cell bank) were tested only by the *-labeled test methods.

| Groups     | Number of nude mice injected (female) | Nodule at the injection site | Tumors beyond the inoculation sites |
|------------|---------------------------------------|-------------------------------|------------------------------------|
| Hela cells | 10                                    | 10                            | 0                                  |
| CCRC-1 (P5)| 10                                    | 0                             | 0                                  |
| CCRC-1 (P15)| 10                                   | 0                             | 0                                  |
| CCRC-1 (P30)| 10                                   | 0                             | 0                                  |

Table 2. Numbers of animals injected with either Hela or CCRC-1 cells and the corresponding tumor incidence.
U/ml and 169.2 of 0.1, 0.01 or 0.001 MOIs, the antigen contents achieved in CCRC-1 cells were 577.0 obtained in CCRC-1 cells than in both MRC-5 and Vero cells (Fig. 6). At day 6 after infection with EV71/SH06 viruses under the same serum-free growth condition. As a result, a significantly higher EV71 antigen content was especially for human use, we then determined in all three cell lines the replication activity of EV71 and Rubella respectively.

The CCRC-1 cells were susceptible to infection of a wide spectrum of viruses. Both MRC-5 and Vero cells are highly permissive to infection of various viruses and are commonly used in production of different viral vaccines. To compare the permissiveness of CCRC-1 to viral infections in parallel with MRC-5 and Vero, all three cell lines were infected with various viruses for 6 days. As determined by the appearance of CPE, it was seen that CCRC-1 cells exhibited a similar profile of viral permissiveness as MRC-5 cells with both showing the susceptibility to the infection of EV71/SH06, MV/Chang-47, RUV/RA27/3, VZV/Oka, SEV, ADV-5 and HSV-2. No apparent CPE was induced by JEV/SA14-14-2 in both CCRC-1 and MRC-5 cells. Meanwhile, Vero cells exhibited a significant susceptibility to all infections but not VZV/Oka (Fig. 3).

CCRC-1 cells are permissive to high viral replication. Using EV71 and Rubella viruses as two representatives, we further determined whether the CCRC-1 cells are permissive to high viral replication for evaluating its ability to achieve high efficiency in manufacturing viral vaccines. We first compared CCRC-1 with MRC-5 or Vero for the replication activity of EV71/SH06 virus, which has been utilized as a viral strain in the production of the inactivated EV71 vaccine using Vero cells. It was observed that, as indicated by viral titer, all cell lines showed a similar level of high viral replication activity as well as the growth kinetics. The highest titer achieved from CCRC-1, Vero or MRC-5 following the infection with the virus of 0.1 MOI were 6.29 ± 0.19 lgCCID50/ml, 6.53 ± 0.19 lgCCID50/ml and 6.29 ± 0.19 lgCCID50/ml, respectively (Fig. 4A). However, although a similarity was seen in the viral titer, a remarkable difference in the production of viral antigen was observed among all three cell lines. The peak EV71 antigen content achieved in CCRC-1, MRC-5 and Vero following the viral infection were 789.7 ± 122.0 U/ml, 599.7 ± 49.7 U/ml and 828.7 ± 27.7 U/ml, respectively (Fig. 4B).

Similarly, we also compared the replication activity of RUV/RA27/3, an attenuated virus strain used in the development of Rubella virus vaccine using MRC-5 cells, between CCRC-1 and MRC-5 cells. It was observed from the virus replication kinetics that the virus was able to efficiently replicate in both cell lines (Fig. 5). The highest virus titers reached at day 6 in CCRC-1 cells following the infection with the virus of 0.1, 0.01 or 0.001 MOIs were 5.81 ± 0.09 lgCCID50/ml, 5.50 ± 0.02 lgCCID50/ml and 5.63 ± 0.02 lgCCID50/ml, respectively, whereas, that in MRC-5 were 6.13 ± 0.18 lgCCID50/ml, 6.00 ± 0.35 lgCCID50/ml and 5.88 ± 0.35 lgCCID50/ml, respectively.

Given that the serum-free cell culture condition has been widely accepted in the production of viral vaccines especially for human use, we then determined in all three cell lines the replication activity of EV71 and Rubella viruses under the same serum-free growth condition. As a result, a significantly higher EV71 antigen content was obtained in CCRC-1 cells than in both MRC-5 and Vero cells (Fig. 6). At day 6 after infection with EV71/SH06 of 0.1, 0.01 or 0.001 MOIs, the antigen contents achieved in CCRC-1 cells were 577.0 ± 12.1 U/ml, 370.3 ± 2.8 U/ml and 169.2 ± 46.2 U/ml, respectively. However, the corresponding values obtained in MRC-5 cells were 134.3 ± 8.6 U/ml, 120.8 ± 7.5 U/ml and 85.1 ± 16.5 U/ml, respectively, and that in Vero cells were 148.1 ± 9.4 U/ml, 68.5 ± 11.8 U/ml, 5.8 ± 1.8 U/ml, respectively, thus strongly demonstrating that, under the serum-free condition, the CCRC-1 cells achieved a much higher production of EV71 vaccine than both Vero and MRC-5 cells (Fig. 6A). Similarly, under the serum-free condition, a significantly higher RUV/RA27/3 titer at day 6 was achieved in CCRC-1 cells than in MRC-5 cells (Fig. 6B). These data demonstrated that the CCRC-1 cells represent a novel high-yielding cell substrate for viral vaccine production with even a greater yield when the serum-free condition was employed.

The EV71 vaccines derived from all three cell lines exhibited a similar immunogenicity. The immunogenicity elicited by vaccines in testing animals as commonly represented by the NAb and NAb conversion rate has been employed to predict clinical efficacy of the relevant vaccines. To evaluate the immunogenicity, the NAb and NAb conversion rates in all mice inoculated with the vaccines generated from different cell substrates were measured. Surprisingly, the immunogenicity in all vaccine-inoculated animals reached almost 100.0% at day 14 after immunization and was persistent at the same level at day 28. The Geometric Mean Titters (GMTs) of the
anti-EV71 NAb elicited by CCRC-1/EV71, MRC-5/EV71, Vero/EV71, CCRC-1/EV71 + Al, MRC-5/EV71 + Al and Vero/EV71 + Al were 1:21.0, 1:19.1, 1:23.7, 1:37.6, 1:31.5, 1:26.2 and 1:18.0, 1:16.4, 1:20.6, 1:21.9, 1:25.1, 1:21.6 at day 14 and 28 post-inoculation, respectively (Fig. 7). These data indicated that, based on the induction of NAb, the vaccines produced in CCRC-1 exhibited the same immunogenic properties as the vaccines generated in MRC-5 cells or Vero cells.

The EV71 vaccines produced in CCRC-1 cells caused no systemic toxicity. To evaluate the existence of any possible systemic toxicity elicited by the EV71 vaccines derived from different cell lines with or
without aluminum adjuvant, the testing animals were monitored for weight change as well as other adverse findings in a three day interval over the entire observation period. The results showed that there was no significant difference in weight change among different testing groups (Fig. 8A). In addition, no other adverse findings, such as fur ruffling, abnormal behaviors, and feeding death or other unexpected death, were observed during the entire observation period. Furthermore, no pathological findings in the heart, liver, spleen, lungs, kidney or muscle were observed in all testing animals, thus suggesting that the EV71 vaccines produced from CCRC-1 cells like that from MRC-5 and Vero cells did not induce any detectable systemic toxicities (Fig. 8B).

The hUC-MSCs lines derived from different donors exhibited an overall high efficiency in viral replication. To determine the viral replication efficiency in hUC-MSCs derived from different donors, we compared the replication efficiencies of EV71-SH06 and RUV/RA27/3 in six hUC-MSC cell lines, i.e. CCRC-1, -2, -3, -4, -5, -6 lines, which were derived from 6 individual donors. The cells of each line were infected with 0.1 MOI EV71-SH06 virus or RUV/RA27/3 virus. The EV71 virus antigen content or Rubella virus titer tested at day 6 after infection was employed to represent the replication efficiency of the relevant virus. The results showed that, for EV71 virus, a relatively higher virus content was obtained in CCRC-1, -2 and -3 lines, among which the...
highest one was CCRC-1 (789.7 ± 122.0 U/ml). Whereas, for Rubella virus, 5 lines, i.e. CCRC-1, -3, -4, -5 and -6, produced a relatively high virus titer with CCRC-3 being the highest one (6.17 ± 0.26 lgCCID50/ml), thus demonstrating that hUC-MSCs as a group exhibited an overall high efficiency in viral replication, although the variations existed among the individual hUC-MSCs (Fig. 9).

**Discussion**

Vaccines have proved to be the most efficacious and cost-effective intervention for assuring public health, and have contributed to a dramatic reduction in the spread and burden of various infectious diseases. Cell substrate is among the most critical components imposing dramatic effects on both quality and quantity of viral vaccine productions. In terms of safety issues regarding tumorigenicity, HDCs represent the safest one among all types of cell substrates. However, because of the limitation in propagation capacity, the continuous diminishing of early passage cells, such as the continuous reduction of the WHO MRC-5 Reference Master Cell Bank, and the fact that all existing HDCs were derived from the fetal lung tissues, which are associated with both technical and ethical issues, searching for new source of derivation and establishing novel HDCs is often needed. Following the previous observation, we provide further evidence that the human umbilical cord rich in mesenchymal stem cells can serve as a more feasible source of HDC derivation and CCRC-1 derived from the human umbilical cord represents a novel high-yielding HDC cell line with a likely higher efficiency than the existing HDCs for the production of at least EV71 vaccines.

The quality of cell substrates is directly associated with the quality and safety of resultant products. Based on major quality requirements of cell substrates, both World Health Organization (WHO) and Chinese pharmacopeia provide a set of guidelines for cell bank characterization for different cell substrates, including HDCs. The major quality requirements from the guidelines for HDCs include the freedom from adventitious agents and non-tumorigenicity.

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Figure 5. Replication activity of Rubella virus in CCRC-1 and MRC-5 cells. CCRC-1 and MRC-5 cells were infected with RUV/RA27/3 virus at MOIs of 0.1, 0.01 and 0.001, respectively. The virus titers were tested every 24 h post infection for 6 days, which were quantified using the standard CCID50 assay and expressed as lgCCID50/ml. Data are shown as the mean ± SD of three independent experiments.

Figure 6. Production of EV71 and Rubella vaccine in CCRC-1, MRC-5 and Vero cells under the serum free culturing condition. The viral suspension collected from the cells, maintained in serum free MEM and infected separately with EV71/SH06 or RUV/RA27/3 virus at MOIs of 0.1, 0.01 and 0.001, were measured for either antigen content or viral titer at day 6 after virus inoculation. (A) EV71 antigen contents obtained in the CCRC-1 cells, MRC-5 or Vero cells. The viral antigens were detected using a quantitative ELISA assay kit and expressed as units/ml (U/ml). (B) The production of Rubella virus titers in CCRC-1 cells and MRC-5 cells. The viral titers were determined using the standard CCID50 assay and expressed as lgCCID50/ml. Data are represented as the mean ± SD of three independent experiments. **P < 0.01, relative to MRC-5 or Vero.
The contamination from adventitious agents in cell substrates represents the most important safety issue associated with cell substrate-derived vaccines. It can cause extremely serious consequences not only to biopharmaceutical enterprises with huge monetary losses, but also to vaccinees with severe health losses. It can happen via a variety of routes including previously undetected contamination of cell substrates themselves, the route from starting materials or from improper operations. In the present studies, we demonstrated clearly that the established CCRC-1 banks were free from the contamination of adventitious agents, thus suggesting that they fully meet the requirements of cell substrate for production of viral vaccines.

The main concerns associated with tumorigenicity of cell substrates can be addressed by testing their potential formation of tumor allografts in immuno-compromised animals, or by testing the potential oncogenicity derived from likely oncogenic components of cell substrates themselves, the route from starting materials or from improper operations. In the present studies, we demonstrated clearly that the established CCRC-1 banks were free from the contamination of adventitious agents, thus suggesting that they fully meet the requirements of cell substrate for production of viral vaccines.

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passage levels, i.e. passage 30, can still keep a similar growth rate and reach to the density pattern as low-passage cells (Fig. 2), thus suggesting that, in terms of the growth density feature, the CCRC-1 cell line is superior to MRC-5 line for vaccine production, which may be beneficial for shortening the production cycles and improving the yield of viral vaccines.

In order to meet the needs of producing viral vaccines of large variety, the cell substrate should have a broad spectrum of viral susceptibility. The high susceptibility to the infection of a vast variety viruses is shared by the

Figure 8. Determination of general toxicity and potential adverse effects for EV71 vaccine produced by CCRC-1, MRC-5 and Vero cells. The general toxicity and potential adverse effects EV71 vaccines produced from the cells with (CCRC-1+Al, MRC-5+Al and Vero+Al) or without (CCRC-1, MRC-5 and Vero) aluminium adjuvant were evaluated in BALB/c mice. PBS (control) and PBS plus aluminium adjuvant (Al) were used as the controls. (A) Weight curve of the vaccine-immunized mice, which were plotted at 3-day intervals. Data are represented as the mean ± SD of the animals, n = 10. (B) Histochemical analysis of tissue specimens from different groups; scale bar = 100 μm.
most frequently used cell substrates, such as MRC-5 cells and Vero cells, thus should serve as a prerequisite feature for novel cell substrates. In this study, we revealed that CCRC-1 shares a similarly high susceptibility to the infection of various viruses with MRC-5. In addition, as another important necessity for virus replication, the cell substrate has to be competent for achieving high yield viral production. Consistent with the finding revealed in the previous study, we found that both EV71/SH06 and RUV/RA27/3 could replicate to high levels in CCRC-1 cells. Importantly, under the serum-free culturing condition, the production of both EV71 and Rubella vaccines in CCRC-1 cells was significantly higher than that in MRC-5 or Vero cells. This high compatibility to viral replication is believed to be attributable to the unique stemness of CCRC-1 cells, which probably makes the cells surviving longer in serum-free culturing condition. This finding is highly relevant to the future vaccine production as the serum-free culturing conditions can better represent the production of EV71 and Rubella virus, respectively, in different hUC-MSC strains (CCRC-1, CCRC-2, CCRC-3, CCRC-4, CCRC-5 and CCRC-6). The measurements were performed at day 6 after infection with EV71/SH06 or RUV/RA27/3 at MOI of 0.1. The yields of EV71 antigen contents (A) or Rubella virus titers (B) are presented and the data are expressed as the mean ± SD of three independent experiments.

In conclusion, we further prove that the CCRC-1 cell line, which may represent a novel category of HDCs, is significantly superior to the traditional cell substrates in safety, cell derivation and viral production. Given the continuous diminishing of the existing HDCs and difficulties in deriving new HDCs from human fetal lung tissues, the findings revealed in this study is very important because it provides a fundamental and efficient solution to solve the problems associated with the existing HDCs. More realistically, through this study, a three-tiered CCRC-1 bank system has been successfully established and fully characterized according to the industry standards and may be served as a more efficient HDC line than MRC-5 at least in the production of EV71 and Rubella viral vaccines. With the existing evidence and new upcoming investigations, the potential value of CCRC-1 in viral vaccine industry may be realized in the near future.

Figure 9. Production of viruses in different hUC-MSC strains. Antigen contents and virus titers were used to represent the production of EV71 and Rubella virus, respectively, in different hUC-MSC strains (CCRC-1, CCRC-2, CCRC-3, CCRC-4, CCRC-5 and CCRC-6). The measurements were performed at day 6 after infection with EV71/SH06 or RUV/RA27/3 at MOI of 0.1. The yields of EV71 antigen contents (A) or Rubella virus titers (B) are presented and the data are expressed as the mean ± SD of three independent experiments.
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B.-Z.Y. proposed and supervised the study, P.C., B.-Z.Y. and J.Z.W. designed the study, P.C. and B.-Z.Y. completed the study, P.C. conducted all experiments, P.C., B.-Z.Y., K.-H.Z., T.N., L.W., W.-D.Y. analyzed data, P.C., B.-Z.Y., J.-Z.W. wrote the paper, and all authors reviewed and approved the manuscript.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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