

1 **A senescence-like cellular response inhibits bovine ephemeral fever virus prolif-**  
2 **eration**

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13

14 **Abstract**

15 During industrial scale production of virus for vaccine manufacturing, antiviral  
16 response of host cells can dampen maximal viral antigen yield. In addition to interferon  
17 responses, many other cellular responses such as the AMPK signaling pathway or se-  
18 nescence-like response may inhibit or slow down virus amplification in the cell culture  
19 system. In this study, we first performed a Gene Set Enrichment Analysis of the whole-  
20 genome mRNA transcriptome and found a senescence-like cellular response in BHK-  
21 21 cells when infected with bovine ephemeral fever virus (BEFV). To demonstrate that  
22 this senescence-like state may reduce virus growth, BHK-21 subclones showing vary-  
23 ing degrees of senescence-like state were infected with BEFV. Results showed the  
24 BHK-21 subclones showing high senescence staining could inhibit BEFV replication  
25 while low senescence-staining subclones are permissive to virus replication. Using a

1 different approach, a senescence-like state was induced in BHK-21 using a small mol-  
2 ecule, camptothecin (CPT), and BEFV susceptibility was examined. Results showed  
3 that indeed CPT-treated BHK-21 is more resistant to virus infection. Overall, these re-  
4 sults indicate that a senescence-like response may be at play in BHK-21 upon virus  
5 infection. Furthermore, cell clone selection and modulating treatments using small mol-  
6 ecules may be tools in countering anti-viral responses.

7

8

9 **Key words:** anti-viral response, senescence-like response, viral vaccine production,  
10 BEFV, Rhabdoviridae, GSEA, BHK-21, bioreactor

11

## 1 **Introduction**

2       Successful virus production using cell cultures depends in large part on the re-  
3 sponse of the host cells. Some cell lines, such as CHO, are highly resistant to virus  
4 infection due to their strong anti-viral interferon response. In contrast, some cell lines,  
5 such as Vero and BHK-21, exhibit low interferon response and are therefore more ame-  
6 nable to virus production for purposes such as vaccine manufacturing (1–3). Upon virus  
7 infection, in addition to interferon responses, a host of other types of responses may  
8 also come into play inside the host cell: senescence-like response, the AMPK signaling  
9 pathway, or innate immune responses that can inhibit or slow down virus amplification.  
10 These anti-viral responses may be further enhanced in the large-scale bioreactor be-  
11 cause of the multiple amplification of the production cells in the culture vessel. To un-  
12 derstand and manage anti-viral responses of the host cell are critical steps for the de-  
13 velopment of an optimal virus culturing system.

14       Bovine ephemeral fever virus (BEFV) belongs to the *Rhabdoviridae* family with  
15 a single-stranded, negative sense RNA genome that encodes five structural proteins (N,  
16 P, M, G, and L) and a nonstructural proteins (4–7). Although mortality due to BEFV  
17 infection is low, reduced milk production during pandemic periods can result in signif-  
18 icant economic losses for dairy farms (8). While inactivated BEFV vaccines provide  
19 sufficient protection, immunity decreases rapidly and cattle need to be vaccinated every  
20 one to two years (9). Therefore, a stable production of this virus as vaccine antigen is  
21 important for the cattle industry.

22       BHK-21 is widely used for BEFV production because of its high virus suscepti-  
23 bility. Clear cytopathic effect (CPE) and cell death can be observed 48 hours after BEFV  
24 infection. However, some BHK-21 cells appear not to show CPE, remain alive, and can  
25 even be passaged. The survival of these cells could mean a less efficient in virus pro-  
26 duction system, thus, it is important to explore potential explanations for the survival

1 of some BHK-21 cells after BEFV infection. In the previous studies, not only the foot-  
2 and-mouth disease virus but also the rabies virus had reported the persistent infection  
3 in BHK-21 cells (10, 11). We also noticed that the BEFV persistent infected BHK-21  
4 cells become enlarged and flattened morphology which similar to the senescence cells  
5 described by the other researchers (12, 13).

6 In this study, we first performed a cell functional analysis of BEFV-infected BHK-  
7 21 cells to look for gene sets are enriched after infection. Enriched gene sets may point  
8 to potential pathways of anti-viral response. We then hypothesized that a senescence-  
9 like state may play a role in virus-resistance of some BHK-21 cells. As supporting evi-  
10 dence, we performed two experiments: 1) subclones of BHK-21 were stained for se-  
11 nescence-like state and their resistance to BEFV infection was studied, and 2) a senes-  
12 cence-like state was induced in BHK-21 using a small molecule, camptothecin (CPT),  
13 and BEFV susceptibility was examined.

14

## 15 **Materials and methods**

16

### 17 **BEFV culture and titrating**

18 Baby Hamster Kidney-21 cells (BHK-21, BCRC#60041, purchased from the Bio-  
19 resource Collection and Research Center, Taiwan) were cultured in growth medium  
20 (MEM, Gibco co. LTD, USA, with 10% FBS, Invitrogen) at 37°C in an atmosphere of  
21 5% CO<sub>2</sub>. The BEFV strain Tn88128 isolated in Taiwan (4) was cultured in BHK-21  
22 cells (MOI = 0.01) in 2% FBS media (MEM media containing 2% FBS) at 37 °C with  
23 5% CO<sub>2</sub>. The mock group cells were incubated with the 2% FBS media only. Virus titer  
24 was determined by the TCID<sub>50</sub> or q-RTPCR method. For TCID<sub>50</sub>, CPE was observed  
25 under the microscope 3 days after inoculation and calculated by using the Reed and  
26 Muench method (14). For q-PCR analysis of virus titer, viral RNA was isolated with

1 QIAzol Lysis Reagent (Qiagen Co. LTD, USA). After reverse-transcription by the  
2 SensiFAST™ cDNA Synthesis Kit (Bioline, Memphis, USA), cDNA samples were pre-  
3 pared with the QuantiFast SYBR Green PCR Master Mix reagent (Qiagen, Hilden, Ger-  
4 many) with the detection primers (BEFV-G F: 5'-TACCCTCCTGCTG-  
5 GATGCTTTTG-3' and BEFV-G R: 5'- CTGTGTGCATTCTAAAACCTGGC -3').  
6 Real-time PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 15 s,  
7 60°C for 30 s and 72°C for 15 s. The virus titer was determined by comparing to the  
8 standard virus stock and shown as TCID<sub>50</sub>/ml.

9

#### 10 **Cell functional analysis for surviving BHK-21 cells after BEFV infection (Gene Set** 11 **Enrichment Analysis, GSEA)**

12 BHK-21 cells were inoculated with BEFV and total cellular mRNA were harvest  
13 at the indicated time with the QIAzol Lysis Reagent (Qiagen Co. LTD, USA) according  
14 to the protocol of the manufacturer. Total cDNA was prepared by the SensiFAST™  
15 cDNA Synthesis Kit (Bioline, Memphis, USA) and analyzed by next generation se-  
16 quencing service (Illumina, 150pb pair end, 6G, Tools co. LTD. Taiwan). All NGS sam-  
17 ples were aligned to the BHK-21 genome (*Mesocricetus\_auratus*. MesAur 1.0.98) by  
18 using of the STAR aligner (15, 16). The gene expression were determined as FPKM by  
19 using the Cufflinks (17) to assemble and quantify transcripts FPKM. To evaluate bio-  
20 logical pathways altered between BEFV-infected BHK-21 cell and mock-infected  
21 BHK-21, the Gene Set Enrichment Analysis (GSEA) (Broad Institute, Cambridge, MA,  
22 java GSEA Desktop Application version 4.1.0) (18, 19) tool was employed. The KEGG  
23 pathway gene subsets of GSEA Molecular Signatures Database (MSigDB) (18) C2-  
24 curated gene sets (186 gene sets) along with external senescence-related gene subsets,  
25 were applied to enrichment analysis (total 204 gene sets). The external senescence-re-

1 lated gene subsets were added in the analysis as the references described in **Supple-**  
2 **mentary table 1**. Gene set enrichment was considered significant at  $FDR < 0.25$  (18).

3

#### 4 **BHK-21 subclones isolation**

5 To isolate subclones of BHK-21, parental BHK-21 cells were trypsinized and re-  
6 suspended in growth media for calculation. The resuspended cells were diluted into 1  
7 cell/0.1ml density and seeded into 96-well plates (corning, co. LTD, USA) (0.1 ml/well).  
8 Confluent cells were then subcultured into 24-well and 6-well plates (corning, co. LTD,  
9 USA) sequentially. Subclones were then stored in freezing media (growth media with  
10 7% DMSO) at  $-80^{\circ}\text{C}$  for further use.

11

#### 12 **Senescence-like-associated SA- $\beta$ -galactosidase staining**

13 SA- $\beta$ -gal staining was chosen as the senescence marker (20) and performed ac-  
14 cording to a protocol published by Dimri *et al.* 1995 (21). Cells were washed twice with  
15 PBS and fixed by 4% paraformaldehyde for 5 min at room temperature and washed  
16 with PBS twice. The fixed cells were stained with the SA- $\beta$ -Gal staining solution (1  
17 mg/ml X-Gal in dimethylformamide, pH 6.0 citric acid/sodium phosphate buffer, 5mM  
18 potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2mM  $\text{MgCl}_2$ )  
19 and incubated at  $37^{\circ}\text{C}$  for 12~16 hours.

20

#### 21 **Dynamic monitoring of cell adhesion by iCELLigence<sup>TM</sup>**

22 The RTCA iCELLigence<sup>TM</sup> system (ACEA Bioscience, Inc., San Diego, CA, USA)  
23 records impedance in real-time and converts into cell index (CI) (22). This can quickly  
24 analyze cell adhesion ability and cell size during cell proliferation. The LS-BHK-21,  
25 BHK-21-parental and HS-BHK-21 cells were loaded into E-Plate L8 (ACEA Biosci-  
26 ences, Inc.) at  $2 \times 10^4$  cells/well and the CI was recorded at 5-minute intervals for the

1 first 12 hours and then at 15-minute intervals till the end of the measurement period.  
2 This is convenient to monitor the cell adhesion ability and cell events during the culture  
3 period, which can clearly distinguish different subclonal cells. The two subclonal cells  
4 were analyzed by iCELLigence equipment and to compare with the BHK-21 parental  
5 cells.

6

### 7 **Relative cell viability calculation**

8 LS-BHK-21, BHK-21-parental and HS-BHK-21 cells were seeded in 6-well plates  
9 1 day before experiment. At about 60% confluence, the subclones were infected with  
10 BEFV (MOI=0.01) and the mock group was incubated with MEM media only. After 48  
11 hours, all experimental groups were trypsinized and counted by hemocytometer under  
12 the microscope. Relative cells viability was described by the following equation: rela-  
13 tive cells viability = (virus infected group/ mock group) × 100%

14

### 15 **Camptothecin (CPT)-induced cell senescence-like response**

16 Cells ( $2 \times 10^5$ /per well) were seeded in 6 well plates and cultured overnight. CPT  
17 were diluted in culture media (250nM final concentration) and added into the cells.  
18 After a 24-hour treatment, cells were infected BEFV at an MOI of 0.01. After indicated  
19 incubation time, the cell images, virus supernatant, cell lysate or total mRNA were har-  
20 vested. The virus supernatants were tittered by TCID<sub>50</sub>.

21

### 22 **Western blot analysis for BEFV N protein**

23 The cells were lysed in the RIPA solution (Tools, co. LTD, Taiwan). The protein  
24 samples were determined by BCA protein assay (Pierce, Rockford, IL, USA), resolved  
25 by SDS-PAGE (20μg/well), and transferred onto a polyvinylidene difluoride membrane

1 (PerkinElmer, MA, USA). After blocking in Hyblock 1min Blocking Buffer (GOAL-  
2 BIO, Taiwan) for 1 min at room temperature and washed by PBST (PBS containing  
3 0.05% Tween-20), the membrane was blotted with mouse anti-BEFV N protein antise-  
4 rum (1:1000) (23). After PBST wash, the membrane was incubated with goat anti-  
5 mouse IgG-HRP (1:5000) antibody. The signal was developed by the ECL plus Western  
6 Blot Detection Reagents (GE Healthcare, WI, USA).

7

### 8 **qRT-PCR for senescence related gene expression**

9 Cellular total RNA was harvest and isolated by QIAzol Lysis Reagent (Qiagen,  
10 Hilden, Germany). Two micrograms of total RNA was reverse-transcribed with Sensi-  
11 FAST™ cDNA Synthesis Kit (Bioline, Memphis, USA). The amounts of p16, p21, IL-  
12 6 and  $\beta$ -actin cDNA were determined by QuantiFast SYBR Green PCR Master Mix  
13 (Qiagen, Hilden, Germany) at the conditions: 95°C for 3 min and 40 cycles of 95°C for  
14 15 s, 60°C for 30 s and 72°C for 15 s. Primers for each target gene were: p16-F:5'-  
15 TCTTGAAACTCTGGCGATA-3', p16-R: 5'-GAAGTTACGCCTGCCG-3', p21-  
16 F: 5'-AGTGTGCCCGCCGTCTCT-3', p21-R: 5'-ACACCAGAGT GCAGGACAGC-3',  
17 IL-6-F: 5'-GTCGGAGGTTTGGTTACATA-3', IL-6-R: 5'-ATCTGGACCCTTTAC-  
18 CTCTT-3' and  $\beta$ -actin -F: 5'-CCAAGGCCAACCGTGA AAAG-3',  $\beta$ -actin-R: 5'-  
19 TGGCCATCTCTTGCTCGAAGTC-3'. The threshold cycle (CT) value of each target  
20 gene was normalized to the  $\beta$ -actin gene, and the mock control group using the  $2^{-\Delta\Delta CT}$   
21 method (24).

22

### 23 **Statistical analysis**

24 Experimental data are expressed as mean  $\pm$  sd. Statistical analysis of variance be-  
25 tween groups was performed by the R Statistics software (4.0.3) with agricolae package.  
26 Additional statistical enrichment analysis of GSEA results were performed using the



1 Hypergeometric test to measure the significance. Significant difference of all statistical  
2 tests was set at 0.05 ( $p < 0.05$ ).

3

#### 4 **Results**

5

#### 6 **BEFV-infected BHK-21 cells showed morphological changes and upregulated se-** 7 **nescence-related gene sets**

8 To study BHK-21 cells' response to virus infection, experimental infection with BEFV  
9 was performed. Infected BHK-21 cells show obvious cytopathic effect (CPE) and cell  
10 death. However, some cells survived and became persistently infected, showing en-  
11 larged and flattened morphology with apparently more vacuoles inside (**Fig. 1.** in BEFV  
12 48h). These cells can be passaged (**Fig. 1.** in Persistent BEFV-infected BHK-21 (p3))  
13 and variable levels of BEFV can be detected (**Table 1**). To investigate potential gene  
14 expression changes within the cells after infection, Gene Set Enrichment Analysis  
15 (GSEA) was performed for BHK-21 cells under three conditions: 1) BEFV 24 h.p.i., 2)  
16 BEFV 48 h.p.i., and 3) BEFV persistent, when compared with uninfected BHK-21-  
17 parental cells. Results showed that senescence-related gene subsets were enriched for  
18 all three experimental groups (5, 15 and 9 aging/senescence gene subsets for group 1,  
19 2 and 3, respectively), demonstrating that aging/senescence gene subsets were upregu-  
20 lated in response to virus infection. The regulation of virus infection in cells showed a  
21 discontinuous process. Firstly, immune/adhesion-related gene subsets were regulated  
22 to respond to virus infection after 24 hours (**Fig. 2,** in BEFV 24 h.p.i.). At 48 h.p.i. and  
23 in BEFV-persistent cells, aging/senescence-related gene subsets were upregulated sig-  
24 nificantly (**Fig. 2,** in BEFV 48 h.p.i. and BEFV persistent). These results suggest that  
25 aging/senescence gene subsets upregulation is a potential mechanism for BHK-21 cells  
26 to counter BEFV infection.

1

## 2 **BHK-21 subclones in senescence-like state are more resistant to BEFV infection**

3 In a slightly different approach, we demonstrated that there are subclones of BHK-  
4 21 that show high extent of senescence-like state and are found to be more resistant to  
5 BEFV infection. A total of 36 BHK-21 subclones were established from the parental  
6 cell line using the limiting dilution method. The subclones were then stained with a  
7 senescence-like cellular response marker, SA- $\beta$ -gal, to determine the extent of senes-  
8 cence-like state (**Supplementary Fig. 1**). Two BHK-21 subclones were picked based  
9 on SA- $\beta$ -gal staining levels and named HS-BHK-21 (for high-staining) and LS-BHK-  
10 21 (for low-staining) (**Fig. 3A**). Analysis showed that HS-BHK-21 has higher adhesion  
11 ability and larger cell size while LS-BHK-21 resembles the parental BHK-21 (**Fig. 3B**).  
12 Upon BEFV infection, HS-BHK-21 showed significantly lower virus titer (85%) when  
13 compared to the parental and LS-BHK-21 (**Fig. 3C**), linking higher senescence-like  
14 state with resistance to virus infection.

15 To mimic virus passage during industrial scale virus production, we further inves-  
16 tigated how virus titer may change with serial passage of BEFV in the BHK-21 sub-  
17 clones (**Fig. 4A**). Results showed that, for three passages, BEFV titers remained low in  
18 HS-BHK-21 when compared to LS-BHK-21 and parental BHK-21 (**Fig. 4B**). This in-  
19 dicates that culturing BEFV in HS-BHK-21 cells may further inhibit virus replication  
20 during serial passage. The survival rate of the subclones after each round of virus in-  
21 fection was also tracked (**Fig. 4C**). The three cell populations showed significantly dif-  
22 ferent cell viability after BEFV infection (HS-BHK-21 > BHK-21-parental > LS-BHK-  
23 21). HS-BHK-21 showed remarkable resistance to BEFV induced CPE in passage 3.  
24 The time-lapse CPE image records in three different senescence states also confirmed  
25 the results (<https://youtu.be/bVz9VxudRL0>). These results indicated that culturing  
26 BEFV in HS-BHK-21 cells may further inhibit the virus replication in the following

1 virus passages. A senescence-like cellular function change that may enhance anti-viral  
2 response in BHK-21 cells was discovered.

3

#### 4 **BHK-21 in drug-induced senescence-like state is more resistant to BEFV infection**

5 Since senescence-like state is drug-inducible, we investigated whether BHK-21 in  
6 this drug-induced senescence-like state is more resistant to BEFV infection. The small  
7 molecule, camptothecin (CPT), was employed to induce senescence in BHK-21 cells  
8 in three senescence state. Upon CPT treatment for 24 h, all cells showed enlarged size  
9 and positive SA- $\beta$ -gal staining (**Fig. 5A upper panel**), giving signs of a senescence-  
10 like state. When the treated cells were infected with BEFV, cytopathic effect was sig-  
11 nificantly inhibited compared to the untreated control (**Fig. 5A lower panel**). A 100-  
12 fold decrease in virus titer was observed when compared to the untreated cells (**Fig.**  
13 **5B**). Also, in the CPT-treated cells, BEFV viral protein N was detected long after in-  
14 fection at 48 h.p.i., verses 24 h.p.i. for the untreated cells (**Fig. 6A**). These results illus-  
15 trated that BHK-21 in CPT-induced senescence-like state is much less permissible to  
16 virus infection. At the gene expression level, further analysis was performed with com-  
17 mon senescence-like markers p16, p21, and IL-6 (**Fig. 6B**). Gene expression for p16  
18 was significantly higher in the CPT-pretreated and BEFV 48 h.p.i. groups. For the p12  
19 gene, expression significantly increased in all CPT-treated groups; notably, the expres-  
20 sion was even higher for the BEFV 48 h.p.i. group. IL-6 gene expression was signifi-  
21 cantly higher only after virus infection. Interestingly, p16 and IL-6 gene overexpression  
22 was significant in the CPT-pretreated only after BEFV infection for 48 hours. Overall,  
23 these results suggest that a senescence-like state was induced by CPT in the BHK-21  
24 cells and upon virus infection, further gene expression change may be observed.

25

26

## 1 **Discussion**

2        In this study, we provided a survey of functional gene sets are affected in BHK-  
3 21 upon BEFV infection. Gene set results and cell characteristics together suggested a  
4 senescence-like cellular response to viral infection. We further found that subclones of  
5 BHK-21 with high senescence marker staining are resistant to BEFV infection.  
6 Conversely, BHK-21 subclones with low senescence staining are permissible to virus  
7 replication and therefore may be better suited for large-scale bioreactors.

8        The definition of senescence has been controversial and definitive  
9 characterization of the senescent state may be difficult. When first discovered,  
10 senescence was associated with DNA damage and telomere shortening. More recent  
11 studies found that senescence is a gradually progressing process (25, 26). Senescence-  
12 like characteristics may even reverse course before the cell goes into total functional  
13 arrest and cell death (27). Furthermore, numerous genes are associated with cell  
14 senescence, for example, the p53, p21, p16, and IL-16 (12, 28, 29). In our study, not all  
15 markers were examined and induced senescent state may or may not be true senescence,  
16 and therefore we used the qualifier ‘senescence-like’ cell response.

17        During our serial passaging of BEFV in different BHK-21 subclones, we observed  
18 a dramatic decrease of virus titer for the HS-BHK-21 subclone, which exhibits high  
19 senescence staining. We suspect that the senescence-associated secretory phenotype  
20 (SASP) maybe at play. Senescent cells are know to exhibit SASP, which includes the  
21 secretion of cytokine, chemokine, extracellular matrix protease, growth factor, and  
22 other signaling molecules that can induce senescence in neighboring cells (30). Baz-  
23 Martínez M. and colleagues reported that drug-induced senescent A549 cells showed  
24 SASP and made daughter cells resistant to vesicular stomatitis virus infection (31). In  
25 our study, it is possible that HS-BHK-21 secreted SASP signals into the virus  
26 supernatant and resulted in the inhibition of virus replication in the following culture

1 passages. SASP may be the cause of virus titer instability in cell culture systems,  
2 especially for high-density bioreactor with a large cell amplifying ratio. There are two  
3 ways to solve this problem: the first is the employment of low senescence cells such as  
4 the LS-BHK-21 subclone and the second is to adjust the culture media with anti-  
5 oxidation or anti-aging components.

6

## 7 **Conclusion**

8 We described a senescence-like cell response in BHK-21 after BEFV infection.  
9 BHK-21 subclones showing high senescence staining could inhibit BEFV replication  
10 while low senescence-staining subclones are permissive to virus replication. The sub-  
11 clones can have utility in virus production for vaccine purposes and can be used for  
12 study to develop non-specific anti-viral molecules.

13

14

1 **Declarations**

2 **Consent for publication**

3 Not applicable

4 **Availability of data and material**

5 The datasets used and analyzed during the current study are available from the corre-  
6 sponding author on reasonable request.

7 **Competing interests**

8 The authors declare that they have no competing interests.

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13 **Authors' contributions**

14 HYW designed the study. YJZ performed the virus infection assay, protein analysis  
15 and gene expression. MKH analyzed the total mRNA expression for the cell func-  
16 tional analysis. CAT isolated the subclonal cells. CYC provided the virus and helped  
17 to interpret the data. HCW help to analyze and interpret the data. YJZ, HYW and LTC  
18 wrote this paper. All authors have read and approved the manuscript.

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22

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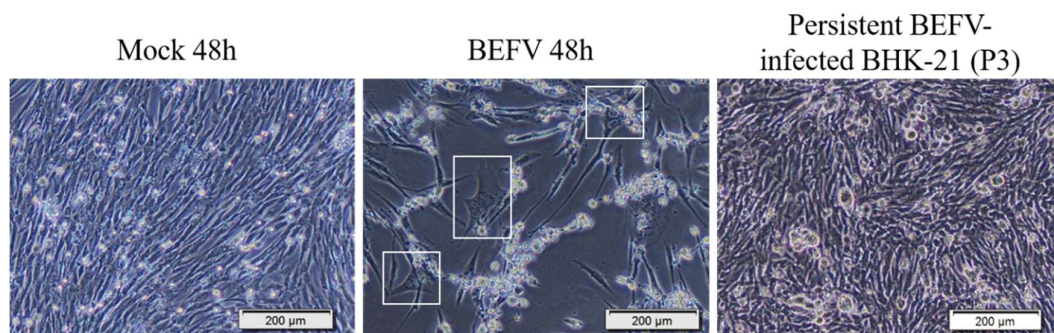
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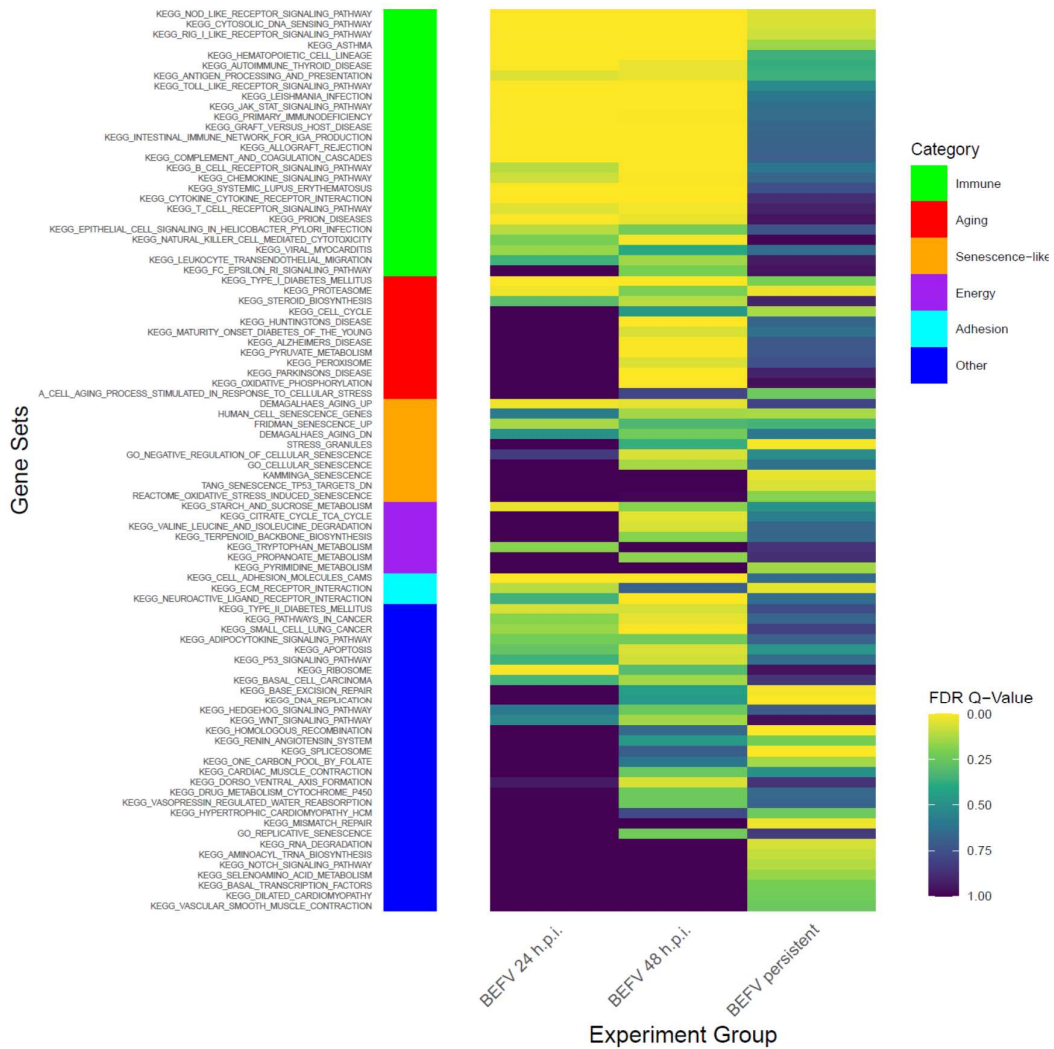
**Table 1. Virus titer of the persistent BEFV-infected BHK-21 cells**

<b>Persistent BEFV-infected BHK-21</b>	<b>TCID<sub>50</sub>/ml</b>
Passage 7 cells	10 <sup>1.8</sup>
Passage 8 cells	10 <sup>5.2</sup>
Passage 9 cells	10 <sup>4.4</sup>
Passage 10 cells	10 <sup>2.1</sup>

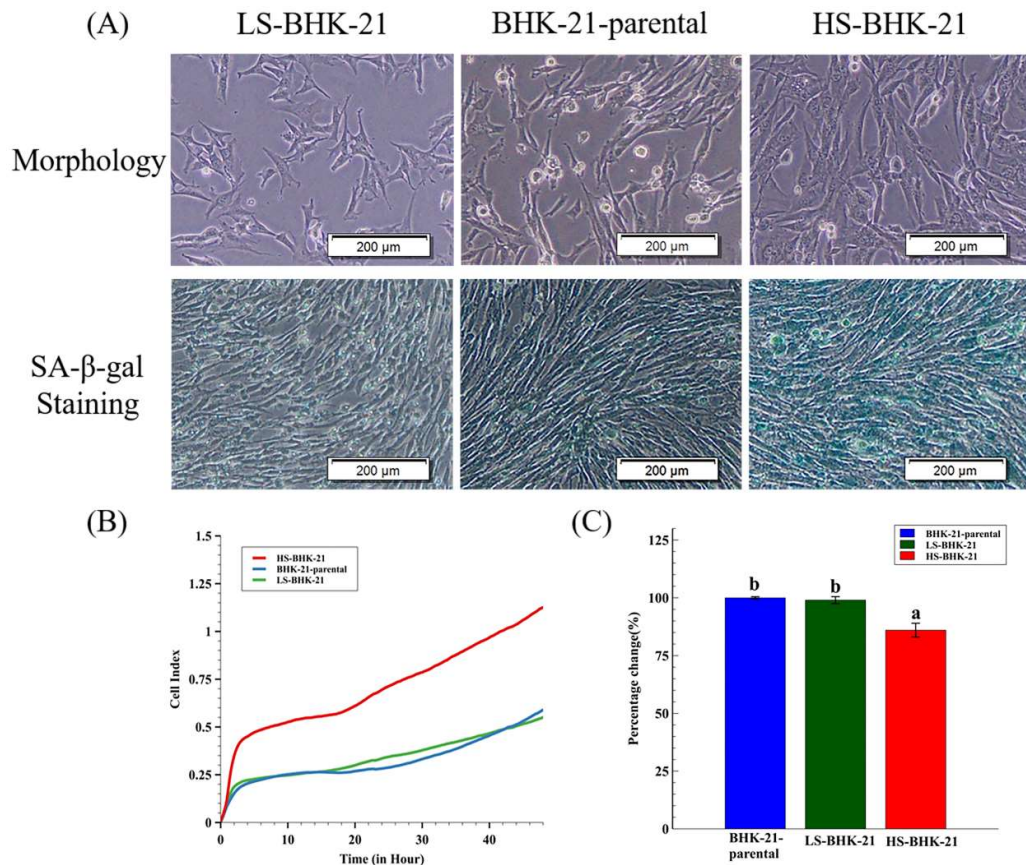
\* The virus titer was determined by qRT-PCR and compare to the standard virus



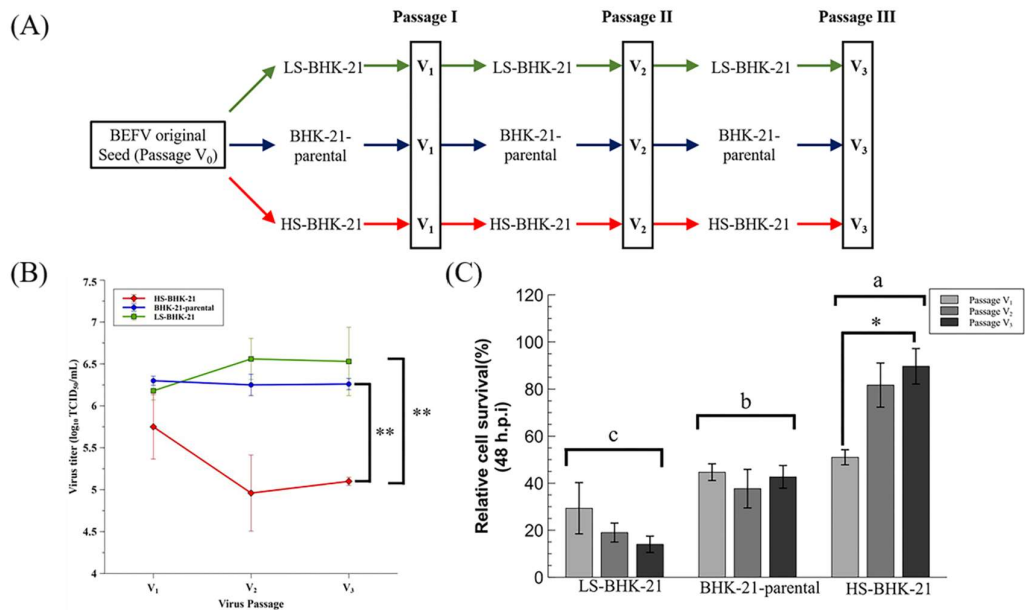
**Figure 1. The BHK-21 Cell morphology changes in BEFV infection.** Mock 48h: The BHK-21 cells were mock infected for 48h. BEFV 48h: The BHK-21 cells were infected with BEFV for 48h. Persistent BEFV infected BHK-21 cells (p3): The BEFV infected BHK-21 cells were survival and keep culture (passaged for 3 times). The cells were imaged with 400x microscope, and the scale bar showed 200μm.



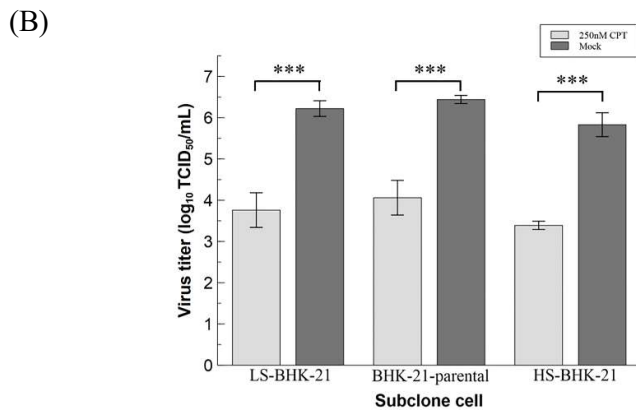
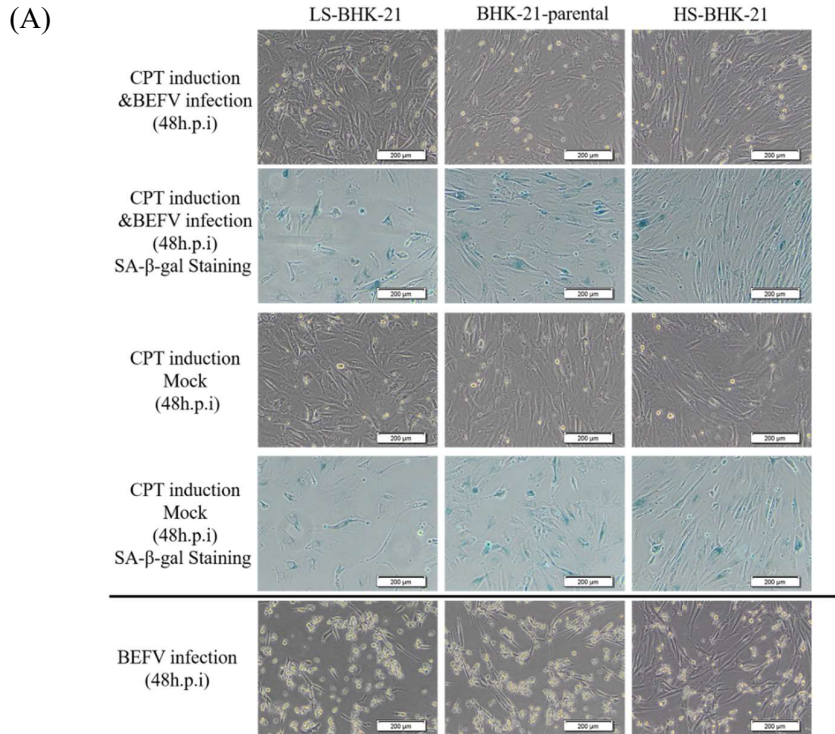
**Figure 2.** The GSEA results show enriched gene subsets in KEGG pathway with senescence related gene subsets. The results demonstrate FDR value ( $< 0.25$ ) in KEGG pathway with senescence gene subset at least significance in one experiment group. Side bar in left show categories of gene subsets.



**Figure 3. The different SA- $\beta$ -gal staining property and the virus production of subclonal BHK-21 cells.** (A) Cell morphology and the SA- $\beta$ -gal staining results for LS-BHK-21, parental and the HS-BHK-21 cells. The cells were imaged with 400x microscope, and the scale bar shows 200 $\mu$ m. (B) The adhesion properties and cell events of the parental and subclonal cells are showed as cell index curve which measured by RTCA iCELLigence™. (C) The particular BEFV titer (48 h.p.i.) cultured by the parental and subclonal cells were compared to the BHK-21-parental group and showed as percentage. The means of percentage change with different letters are significantly different (Tukey's HSD,  $p < 0.05$ ).

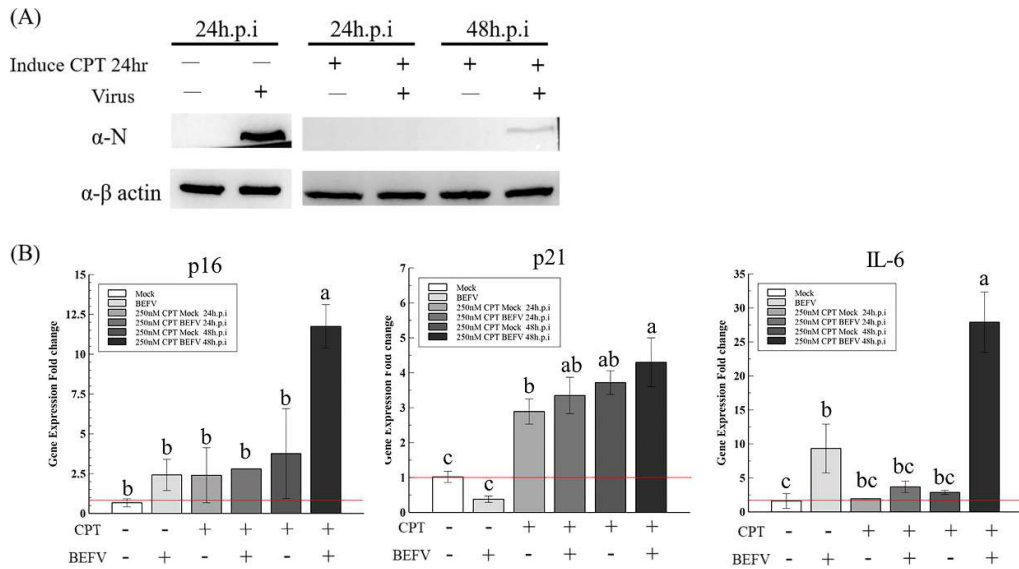


**Figure 4. The virus production ability of the parental and subclonal cells in serial virus passages.** (A) The three serial passages experiment design was showed. (B) The virus supernatant of the three continuous serial passages in three different cells population were harvested at 48 h.p.i., and determined by TCID<sub>50</sub> assay. (C) The relative cell survival percentage of the parental and subclonal cells were determined at 48 h.p.i. by directly trypsinized cell counting, and the percentage was compared to the mock infection respectively. Three independent repeats were performed, and were analyzed by one-way ANOVA. Means with different letters are significantly different (Tukey's HSD,  $p < 0.05$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$  compared to indicated group.



**Figure 5. CPT induced senescence-like cell response inhibited virus replication in BHK-21 parental and subclonal cells. (A)** The parental and subclonal cells were pretreated with CPT (250 nM) for 24hr, and then infected by BEFV or media only as mock control. The senescence-like cell response were determined by SA-β-gal staining and imaged by 400x microscope, and the scale bar shows 200μm. **(B)** The virus titer in the supernatant from CPT pretreatment assay (n=3) were analyzed by TCID<sub>50</sub> and analyzed by t-test. \*\*\*  $p < 0.001$





**Figure 6. The CPT induced senescence-like response markers, P16, P21 and IL-6, in BHK-21-parental cells with or without BEFV infection. (A)** The BHK-21-parental cells were pretreated by CPT for 24hr and followed the BEFV infection for 24 and 48hr as indicated. The virus were detected by Western blotting with the mouse anti-N antiserum. **(B)** The cell p16, p21 and IL-6 gene expression in (A) were analyzed by the qRT-PCR respectively and showed as gene expression fold change compare to mock (red line indicate 1 fold). Means with different letters are significantly different (Tukey's HSD,  $p < 0.05$ ).