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Further Development of an Equine Cell Line that can be Propagated over 100 Times

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Cell lines originating from horses are necessary for isolation and propagation of equine herpesviruses (EHV). Although we established an equine-derived cell line, FHK-Tcl3, propagation ceased after fewer than 40 passages. In this study, FHK-Tcl3 cell propagation continued beyond 40 passages, achieving over 100 passages. FHK-Tcl3 cells were then cloned by limiting dilution at the 100th passage. Cloned cells were termed FHK-Tcl3.1. FHK-Tcl3.1 cells grew well and were propagated every 3 to 4 days by splitting 1:5. In addition, EHV-1, -2 and -4 showed a clear cytopathic effect (CPE) in FHK-Tcl3.1 cells, and this CPE was very similar to those seen in parental FHK-Tcl3 and primary fetal horse kidney cells. FHK-Tcl3.1 cells continue to propagate and the current passage record is over 100 times after cloning. Therefore, this cell appears to have been immortalized. FHK-Tcl3.1 cells have potential for growth and diagnosis of various equine viruses, including equine herpesviruses.

Key words: *cell line, equine herpesvirus*

Equine herpesvirus (EHV) type 1 (EHV-1) can grow well in various cell lines, including equine-, rabbit- and bovine-derived cell lines. However, it has been reported that EHV-1 propagated in non-equine cells possesses a mutation in the glycoprotein C (gC) gene, and is more susceptible to heparin [10]. On the other hand, EHV type 4 (EHV-4) has more restricted host-specificity and grows well only in cells derived from horses, such as primary fetal horse kidney (FHK) cells. Furthermore, EHV-4 adapted to Madin-Darby bovine kidney (MDBK) cells possesses mutations in the gB gene, and is more susceptible to heparin (unpublished data). Therefore, cell lines originating from horses are necessary for mutation-free isolation and propagation of equine herpesviruses.

As cell lines originated from equine are very rare, EHV-4 propagation, isolation and diagnosis are difficult to achieve in a laboratory. Recently, a new equine cell line, FHK-Tcl3, was established at our laboratory [6]. The FHK-Tcl3 cell line is able to support viral growth of EHV-1 and EHV-4, as well as primary FHK cells, and was found to be useful for isolation of EHV type 2 (EHV-2) and EHV-4. EHV type 3 (EHV-3) could also grow in FHK-Tcl3 cells. However, in three experimental trials, it was found that the cells could not be propagated beyond 40 passages.

In this study, we succeeded in propagating FHK-Tcl3 cells beyond 100 passages and found that the cloned cell line, FHK-Tcl3.1, could support the growth of EHV-1, -2 and -4.

FHK-Tcl3 cells were established by transfection of primary FHK cells with plasmid pLNCLT (kindly provided by Dr. S. Yasumoto of the Kanagawa Cancer Institute, Yokohama, Japan) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units of penicillin and 100 μg of streptomycin per milliliter [6]. Although FHK-Tcl3 cells were transformed with the simian virus 40 (SV40) large T antigen, propagation ceased after fewer than 40 passages in propagation

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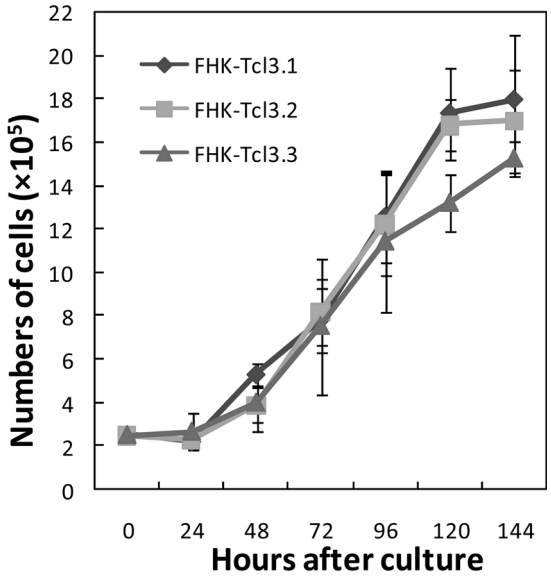

Fig. 1. Growth of cloned cells. Vertical error bars indicate standard deviation of means.

attempts prior to this study. Despite repeated efforts (3 trials), no immortalized cell line could be obtained. At the fourth trial, FHK-Tcl3 cells kept growing after 40 passages and were propagated 100 times. In the 100th passage, FHK-Tcl3 cells were cloned by the limiting dilution method. Briefly, cells were dispensed into a 96-well multiplate (Sumitomo Bakelite, Tokyo, Japan) at a concentration of 0.1 to 10 cells per well. After limiting dilution, three cell clones, termed FHK-Tcl3.1, FHK-Tcl3.2 and FHK-Tcl3.3, were selected, expanded and maintained.

For comparison of cell growth, approximately 2.5×10^5 of each cloned cell line were seeded in 35-mm dishes and cells were collected by treatment with 0.2% trypsin (Nacalai Tesque, Kyoto, Japan) and 0.02% ethylenediamine tetraacetic acid (EDTA) (Wako, Osaka, Japan) every 24 hr. The number of cells was counted using a Burker-Turk hemocytometer after staining with Trypan blue. FHK-Tcl3.1 and FHK-Tcl3.2 cells grew well and their cell numbers (approximately 1.7×10^6) plateaued at 120 hr. FHK-Tcl3.3 cells grew more slowly than the other two lines (Fig. 1). For further studies, FHK-Tcl3.1 cells were randomly selected and propagated every 3 to 4 days by splitting 1:5. After cloning, our established FHK-Tcl3.1 cells grew well and cell growth was similar to that of parental FHK-Tcl3 cells [6]. FHK-Tcl3.1 cells continue to grow at a passage record of 100 times after cloning. Therefore, these cells appear to have been immortalized. Although it is unknown why the FHK-Tcl3 cells continued to grow after the 40th passage, this is a novel report of equine cells being propagated over 200 times.

Previous studies have shown that FHK-Tcl3 cells can support the growth of EHV-1, -2 -3 and -4, and EHV-2 and -4 can be isolated from horses by using FHK-Tcl3 cells [6]. Therefore, the sensitivity of FHK-Tcl3.1 cells to EHVs was also examined. FHK-Tcl3.1 cells were infected with EHV-1 strain 89c25p [4, 7, 8] or EHV-4 strain TH20p [5, 9] at 100 plaque forming units, 200 μl of EHV-3 strain E51129 [6] viral solution or only medium. Cells were observed every day and CPE was recorded using Motic Images plus Ver. 2.1 (Shimazu-rika, Tokyo, Japan). EHV-1, -2 and -4 showed a clear cytopathic effect (CPE) in FHK-Tcl3.1 cells (Fig. 2). This CPE was very similar to those seen in parental FHK-Tcl3 cells and primary FHK cells. These results indicate that the immortalization and cloning of the cells did not change the characteristics of the FHK-Tcl3 cells. Although the ability of FHK-Tcl3.1 cells to support the growth of the other equine herpesviruses, EHV-3 and EHV-5, was not investigated in this study, it is believed that FHK-Tcl3.1 cells will support the growth of EHV-3, as EHV-3 is able to grow in parental FHK-Tcl3 cells. In addition, EHV-5 is likely to grow in FHK-Tcl3.1 cells, as EHV-2 and -5 are closely related gammaherpesviruses [1–3, 11].

In conclusion, the novel equine cell line, FHK-Tcl3.1, which was propagated over 100 times, has successfully been established and can be used for the propagation of equine herpesviruses. In the future, FHK-Tcl3.1 cells may be useful for propagation, diagnosis and research of numerous equine viruses, including equine herpesviruses.

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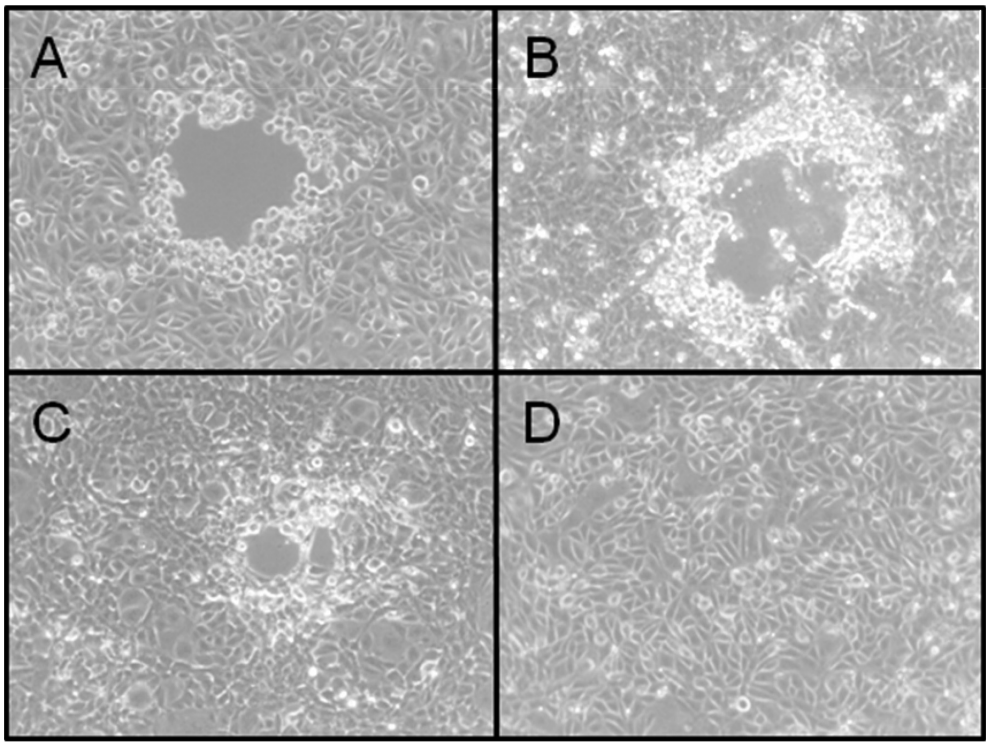

Fig. 2. CPE in FHK-Tcl3.1 cells after infection with EHV-1, -2 and -4. FHK-Tcl3.1 cells were infected with EHV-1 (A), EHV-4 (B), EHV-2 (C) or only medium (D). Cytopathic morphological changes were observed after 32 hr (EHV-1), 72 hr (EHV-4) and 153 hr (EHV-2).

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