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REPORT

Establishment of a novel ovine kidney cell line for isolation and propagation of viruses infecting domestic cloven-hoofed animal species

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Abstract A sheep kidney-derived cell line, FLK-N3, was successfully established after serial (>100) passages. Persistent infection of this cell line with viruses and mycoplasma was not detected. The cells grew well and showed susceptibility to a wide variety of viruses derived from ovine, bovine, and porcine species, including orf virus, maedi visna virus, bovine herpesvirus 1, bovine parainfluenza virus 3, bovine viral diarrhea viruses 1 and 2, bovine coronavirus, bovine respiratory syncytial virus, bovine enterovirus, suid herpesvirus 1, and porcine enterovirus. These results suggest that the FLK-N3 cell line could be useful for isolation and propagation of viruses that affect cloven-hoofed animals.

Keywords Cell line · Establishment · Ovine · Virus propagation

Cells of ovine origin are widely used for various experiments. Madin–Darby ovine kidney (MDOK) cells (Madin and Darby 1958) are popular and commercially available from the American Type Culture Collection. Primary cells from the ovine embryonic kidney and lung seem to be the most useful types of cells for isolation and propagation of sheep viruses. However, in some situations, obtaining fresh primary cells has proven to be difficult. In particular, since the outbreak of bovine spongiform encephalopathy (BSE) was reported in 2001 in Japan, the Japanese government has prohibited using any tissues from cattle aged 2 yr and older, or goats and sheep 1 yr and older. This includes fetuses in these animals, until BSE and scrapie test results have been returned and are negative. The Japanese government has also strongly limited the import of products from cloven-hoofed animal species including sheep cell lines and cells maintained with bovine sera such as MDOK cells.

Many cell lines from various organs are established by introducing the simian virus 40 (SV40) T antigen gene, the E6E7 gene of papillomavirus, or a telomerase gene into primary cells (Le Poole et al. 1997; Inoshima and Ishiguro 2009; Pan et al. 2010). However, in recent years, the development and application of living genetically modified organisms using recombination techniques are regulated and monitored by law (Yamanouchi 2007). Thus, a cell line originating from sheep that has not been subjected to recombination techniques is essential in studying the outbreak of infectious diseases and the cytophysiology of sheep.

We attempted to establish a novel cell line originating from sheep using standard methods and without any genetic recombination techniques. We report the establishment of a novel cell line that shows susceptibility to several viruses derived from sheep and other animal species.

Primary fetal lamb kidney (FLK) cells from the fetus of a normal sheep were maintained in Eagle's minimal essential medium supplemented with 5% of fetal calf serum and 10% tryptose phosphate broth. The cells were successfully cultured over 100 serial passages and the cell line designated FLK-N3.

To examine cell growth, approximately 5×10^4 cells were seeded in a 10-cm tissue culture dish. Cells at passage 106 (P106) were collected by treatment with trypsin–ethylenediaminetetraacetic acid (EDTA) at 1, 3, 4, and 7 d of

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Table 1. Propagation of viruses derived from different animal species in FLK-N3 cells

Animal species	Virus	Strain	Culture method	CPE/DPI	Virus titer[a] ($TCID_{50}$/mL)	References
Ovine	ORFV	HIS	Static	+/4	NT	Kanou et al. (2005)
Ovine	ORFV	Iwate	Static	+/2	NT	Kumagai et al. (1971)
Ovine	MVV	M88	Static	+/10	NT	Kindly provided by Dr. Onuma
Caprine	CAEV	N40-8	Static	−	NT	Konishi et al. (2004)
Bovine	BoHV-1	758	Static	++/2	10^5	Nishimado et al. (1972)
Bovine	BVDV	Nose	Static	++/2	$10^{2.5}$	Kodama et al. (1974)
Bovine	BVDV	KZ91CP	Static	++/3	$10^{2.9}$	Nagai et al. (1998)
Bovine	BCoV	Kakegawa	Static	+/2	10^4	Akashi et al. (1980)
Bovine	BCoV	Mebus	Static	+/5	NT	Mebus et al. (1973)
Bovine	BCoV	119WN	Static	−	NT	Tsunemitsu et al. (1995)
Bovine	BPIV-3	BN-1	Static	−	NT	Inaba et al. (1963)
Bovine	BPIV-3	BN-1	Rotary	±/4	NT	
Bovine	BPIV-3	YN-1	Rotary	±/4	NT	Inaba et al. (1963)
Bovine	BPIV-3	910N	Rotary	±/4	NT	Inaba et al. (1963)
Bovine	BRSV	NMK-7	Static	+/2	10^4	Inaba et al. (1970)
Bovine	BRSV	NMK-7	Rotary	±/3	NT	
Bovine	BEV	BF-1	Static	++/3	10^6	Inaba et al. (1960)
Bovine	BEV	C-121E	Static	+/3	10^5	Kurogi et al. (1976)
Swine	SuHV-1	Yamagata S-81	Static	++/1	10^7	Fukusho et al. (1981)
Swine	PEV A (PEV-8)	4CC	Static	−	NT	Honda et al. (1990)
Swine	PEV B (PEV-9)	UKG410/73	Static	+/3	10^4	Knowles et al. (1979)
Swine	PEV B (PEV-10)	W47H	Static	++/3	10^5	Honda et al. (1990)
Swine	PTV-1	SF12	Static	−	NT	Morimoto et al. (1968)
Swine	PTV-2	SFK10	Static	−	NT	Morimoto et al. (1968)
Swine	PTV-4	SF16	Static	−	NT	Morimoto et al. (1968)
Swine	PPV	90HS	Static	±/7	NT	Morimoto et al. (1972)

ORFV orf virus, *MVV* maedi visna virus, *CAEV* caprine arthritis encephalitis virus, *BoHV-1* bovine herpesvirus 1, *BVDV* bovine viral diarrhea virus, *BCoV* bovine coronavirus, *BPIV-3* bovine parainfluenza virus 3, *BRSV* bovine respiratory syncytial virus, *BEV* bovine enterovirus, *SuHV-1* suid herpesvirus 1, *PEV* porcine enterovirus, *PTV* porcine teschovirus, *PPV* porcine parvovirus, *NT* not tested, *CPE* cytopathic effect, ++ prominent, + clear, ± weak, − no effect on the cells, *DPI* days post-inoculation

[a] Virus titer was estimated at 7 DPI

culture and then counted following staining with 0.05% (*w*/*v*) trypan blue diluted in phosphate-buffered saline.

To examine persistent infection of viruses such as ovine herpesvirus-2, alcelaphine herpesvirus-1, bovine viral diarrhea virus (BVDV), bovine leukemia virus, maedi/visna virus (MVV), border disease virus, and mycoplasma in FLK-N3 cells, polymerase chain reactions (PCR) were performed according to previous reports (Hsu et al. 1990; Baxter et al. 1993; Harasawa et al. 1993; Harasawa and Tomiyama 1994; Vilcek et al. 1994; Fechner et al. 1997; Celer et al. 2000). DNAs were extracted from FLK-N3 cells using a Sepa Gene kit (Sanko Junyaku, Tokyo, Japan), and RNAs were also extracted using a Sepa Gene RNA isolation kit (Sanko Junyaku) according to the manufacturer's instructions. Persistent infection by BVDV in the cells was also estimated by an immunoperoxidase method

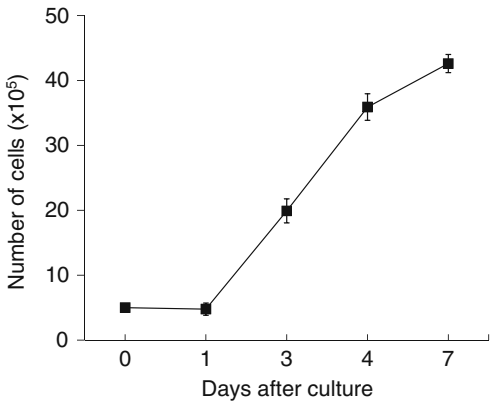

Figure 1. Growth of FLK-N3 cells. Approximately 5×10^5 cells were seeded in 10-cm tissue culture dishes. Cells were collected by treatment with trypsin–EDTA after 1, 3, 4, and 7 d in culture. Then, cells were counted after staining with trypan blue. Data are shown as the means of three independent experiments±SD.

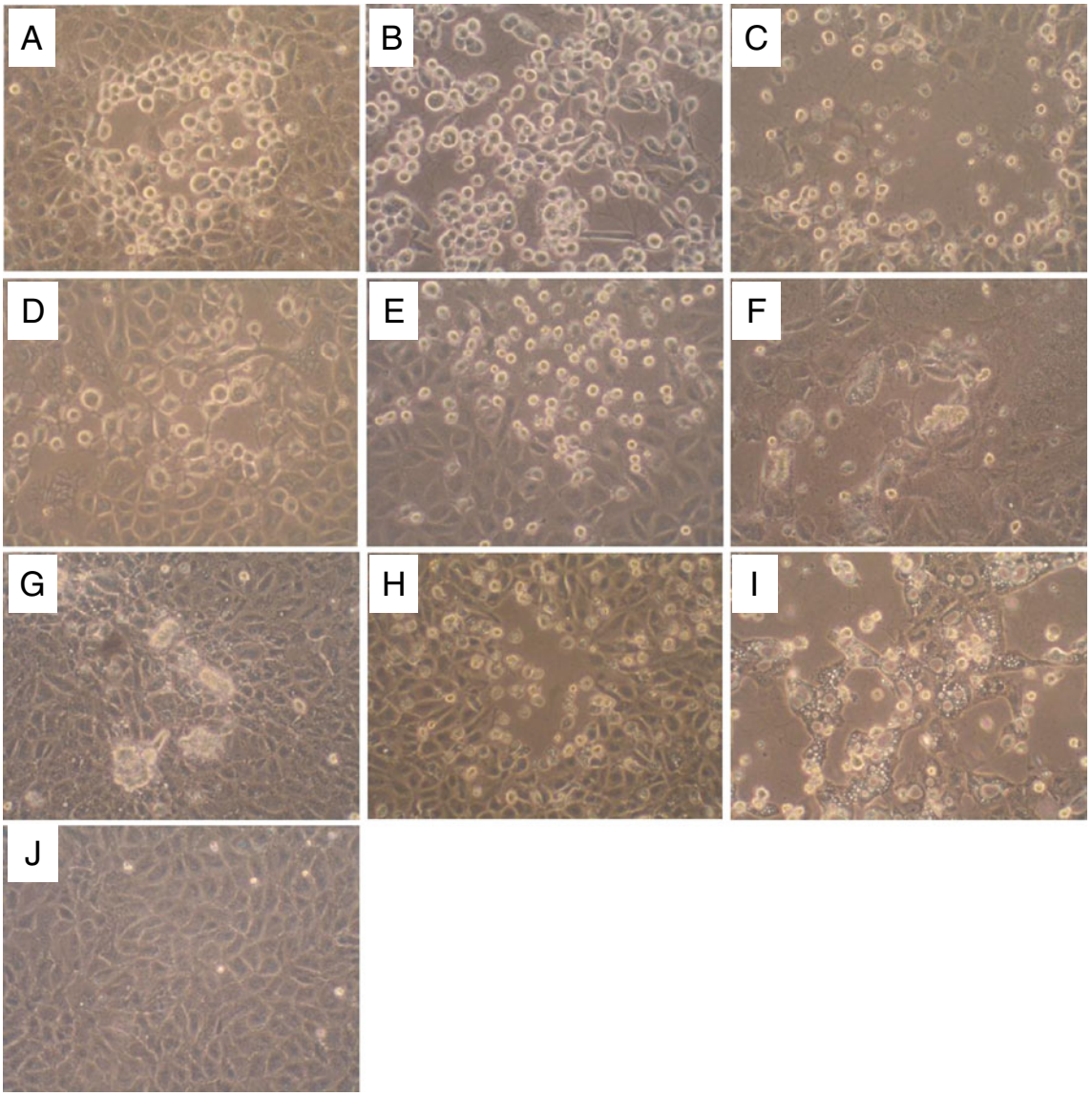

Figure 2. Cytopathic effects on FLK-N3 by virus infection. FLK-N3 cells were infected with ORFV Iwate (*A*), BoHV-1 758 (*B*), BEV C-121E (*C*), SuHV-1 Yamagata S-81 (*D*), PEV B W47H (*E*), BCoV Kakegawa (*F*), BRSV NMK-7 (*G*), BVDV KZ-91CP (*H*), MVV M88 (*I*), and virus-uninfected negative control FLK-N3 cells (*J*). Cytopathic effects were observed at days 1 (*D*), 2 (*A*, *B*, *F* and *G*), 3 (*C*, *E* and *H*), and 10 (*I*) post-infection, respectively. Original magnification, ×100.

that employed a monoclonal antibody against the BVDV NS3 protein (Kameyama et al. 2006).

To investigate the viral susceptibility of FLK-N3 cells, the cells were infected with several viruses derived from ovine, bovine, and swine species (Table 1) at a multiplicity of infection of 0.1. The cells were cultured in a conventional static culture or rotary culture. Cytopathic effect (CPE) of the cells was observed, and the virus titer in the culture supernatant was determined by the Reed–Muench method (Reed and Muench 1938).

The number of cells increased in wells during culture and was almost confluent by day 4 (Fig. 1). The PCR and immunoperoxidase studies clearly showed that no viruses could be detected in the FLK-N3 cells. FLK-N3 cells grew well with no persistent infection observed in the cells.

The viruses used in this study grew well in FLK-N3 cells, grew within almost 7 d after inoculation (Table 1). Representative CPEs of susceptible viruses on FLK-N3 cells are shown in Fig. 2. FLK-N3 cells showed a high susceptibility to orf virus (ORFV), bovine herpesvirus 1 (BoHV-1), bovine enterovirus (BEV), suid herpesvirus 1 (SuHV-1), and porcine enterovirus (PEV) type B. Bovine coronavirus (BCoV) and PEV showed strain specificity or type specificity in the cells. Furthermore, bovine parainfluenza virus 3 (BPIV-3) and bovine respiratory syncytial virus (BRSV) exhibited a

different type of CPE, when static or rotary culturing methods were used. The virus titers at the time of CPE appearance were almost equal to or lower than titers which were propagated in the Madin–Darby bovine kidney, a human rectal adenocarcinoma cell line (HRT-18), and a monkey kidney epithelial cell line (Vero) (data not shown).

FLK-N3 cells had some novel characteristics. For propagation and isolation of BCoV, HRT-18 cells are routinely used in many laboratories (Mebus et al. 1973; Akashi et al. 1981; Tsunemitsu et al. 1995). However, it is well known that the CPE is hard to observe in HRT-18 cells infected with BCoV. In contrast, FLK-N3 cells infected with BCoV clear demonstrated a CPE. Furthermore, observation of CPE because of BVDV infection is normally carried out in primary BFM cells. FLK-N3 cells showed clear CPE after BVDV inoculation. However, the titers of BVDV 1 and BVDV 2 propagated in FLK-N3 were $10^{2.7}$- and $10^{1.6}$-fold less at 7 d post-inoculation, respectively, than that in primary bovine fetal muscle (BFM) cells (data not shown). The titer of other viruses that showed a CPE in FLK-N3 cells was almost the same as that in primary cells. Susceptibility of FLK-N3 cells to BVDV may be lower than that of BFM cells.

MVV showed CPE at day 10, whereas caprine arthritis encephalitis virus (CAEV) did not exhibit any CPE. MVV and CAEV are ovine/caprine lentiviruses (Linial et al. 2005), with MVV showing a CPE and CAEV infection not resulting in any CPE on FLK-N3 cells. This may be dependent on the species susceptibility in FLK-N3 cells.

The cell line established in this study supported the in vitro growth of several viruses of cloven-hoofed animals. This suggested that FLK-N3 cells may be useful in studying general host–parasite relationships. In addition, because viruses derived from different animal species can grow in these cells, preparation of several kinds of cells may not be required. The usage of FLK-N3 cells might lead to less labor intensive and quicker assay results. FLK-N3 cells should be useful for in vitro research and the diagnosis of viral diseases.

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