



A new cell line from goat embryonic kidney may be a candidate to produce influenza virus vaccine

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Received: 31 August 2012; Accepted: 24 March 2015

ABSTRACT

This study established a goat embryonic kidney (GEK) cell line which was susceptible to influenza virus. Kidney cells were separated from fetus of Matou goat and GEK cell line was established by cell biology techniques. Tumorigenicity of GEK cell line was tested by tumorigenesis assay, and its marker protein expression was tested by immunohistochemistry. Receptors of influenza virus and susceptibility to H1N1 influenza virus were also tested by immunofluorescence method and cell infection assay, respectively. Results showed that GEK cells transplanted into BALB/c nude mice could not induce tumorigenesis. Immunohistochemical analysis showed that the cells expressed cytokeratin 18 (CK-18) and vimentin which are marker proteins of renal epithelial cells. Immunofluorescent assay indicated that the abundance of α 2,3-sialic acid (SA) receptor and α 2,6-SA receptor on the surface of GEK cells were higher than that of Madin-Darby canine kidney (MDCK) cells. The influenza virus titers in GEK cells were similar to that of MDCK cells. Therefore, the GEK cell line established here will possibly contribute to the future influenza virus isolation and vaccines development.

Key words: Goat embryonic kidney cell, Influenza virus, Receptor, Tumorigenicity

Influenza vaccination is the most effective method for the prevention and control of influenza. Now, producing influenza vaccine in chicken embryo is the most commonly used method (Hui and Macdonald 2011), which rely on a lot of embryonated eggs. If a pandemic virus is pathogenic for poultry, embryonated eggs may be in short supply (Hannoun 2013). In contrast to the egg-based method, cell culture is more acceptable to influenza vaccine production. Evidences indicated that vaccines derived from cell culture can induce immunity responses as good as egg-derived vaccines (Genzel and Reichl 2009). The development of cell lines suitable for influenza virus propagation has become current research trend. At present, there are only a few kinds of cells that can be used for the preparation of influenza vaccine, including African green monkey kidney (Vero) and MDCK cell lines (Genzel *et al.* 2010, Liu *et al.* 2012). Therefore, development of new cell lines for influenza virus propagation will be beneficial to the influenza vaccine production and virus isolation. Here, we

described the development and characterization of a continuous cell line derived from fetal goat kidney tissue. This cell line is a promising candidate for cultivation of influenza virus and vaccine production.

MATERIALS AND METHODS

Primary cell culture: Matou goat, 3 months pregnant, was deeply anesthetized by 8% chloralhydrate and its fetus was taken out. The animal experiment procedures were performed in accordance with the guide for the care and use of laboratory animals, established by the Centre for Disease Control and Prevention in Hubei Province. Fetal kidney was quickly isolated under sterile condition and washed twice with sterile phosphate-buffered saline (PBS). The GEK cell preparation was performed as per Lash *et al.* (2005). The cells were inoculated to the cell culture flasks and cultured in 10% fetal bovine serum (FBS)-DMEM medium at 37° C with 5% CO₂.

Cloning of GEK cells and subculture: After the GEK cells had covered 90% area of the flasks, they were digested by trypsin. Detached GEK cells were limited diluted and added into 96-well cell culture plates. The wells containing only a pure population of GEK cells were selected, and the cells in these wells were collected. The pure GEK cells were inoculated into new tissue culture flasks and subcultured at 37°C with 5% CO₂. When the rate of cell

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growth was stable, cells were collected and preserved in liquid nitrogen for future use.

Growth properties and chromosome analysis: GEK cells were harvested and resuspended in 10% FBS-DMEM medium, added into the 24-well culture plate with 2.0×10^4 cells in each well. The number of GEK cells in each well was counted and recorded at an interval of 24 h for 8 days. The growth curve was determined according to the average value of cell count at various time points, and the doubling time of GEK cells was also calculated. The chromosomes of GEK cells at 60th passage were prepared according to Fan *et al.* (2007). Chromosome numbers of metaphase cells were counted by microscopy for karyotype analysis.

Tumorigenesis assay: GEK cells suspended in serum-free-DMEM medium were injected into 10 BALB/c nude mice subcutaneously, with a dose of 0.2 ml (containing 1.0×10^7 cells) per mouse. Meanwhile, 10 BALB/c nude mice inoculated with 1.0×10^7 Hela cells (purchased from CCTCC) per mouse were used as positive control. The inoculated mice were observed for 3 months; and their tumorigenic status was monitored daily.

Determination of marker protein expression: Renal epithelial cell marker proteins (Vimentin and CK-18) were tested by immunocytochemistry method (Wise *et al.* 2005).

Analysis of influenza virus receptors on GEK and MDCK cells: The abundance of α 2,3-Sialyltransferase (SA) receptor and α 2,6-SA receptor on GEK cells and MDCK cells were determined using immunofluorescent assay. Briefly, cells in wells of tissue culture plates were incubated with digoxigenin (DIG)-labeled Sambucus nigra agglutinin (SNA) (specific for α 2,6-SA) or Maackia amurensis agglutinin (MAA) (specific for α 2,3-SA) for 1 h at room temperature. After three washes with cold PBS, cells were incubated with fluorescein-conjugated anti-DIG antibody for 1 h at room temperature, followed by 3 washes with cold PBS. The cell samples were examined under a fluorescence microscope.

Virus infection assay: Influenza virus strain A/WS/33 (H1N1) was used to test the viral susceptibility of GEK cells. GEK cells cultured in 24-well plates were incubated with viral solution (0.5 ml, MOI=3) for 1 h at 33°C. The wells were washed with fresh medium to remove unadhered virus. Each well was then added DMEM medium supplemented with 1% FBS and 2 μ g/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. The virus cultures were collected at 24, 36, 48, 60, 72 and 96 h post-infection, respectively. Meanwhile, cytopathic effects (CPE) were examined daily by inverted light microscopy. MDCK cells treated similarly were used as positive control. Virus titers in culture supernatants collected at various times post-infection were determined by hemagglutination assay described previously (Lee *et al.* 2009).

RESULTS AND DISCUSSION

Primary cell culture and subculture: Primary GEK cells were found to attach to the bottom of the flasks 24 h after

cultivation *in vitro*. Most of them were cobblestone-shaped or spindle-shaped. When the cells covered 90% area of the flask, they were divided into two culture flasks at a ratio of 1: 2. After 3 passages, the cells were treated by limited dilution, and a single epithelial-like cell formed colony was selected. After 15 passages, the cells were typical epithelial cells with polygonal shape (Fig. 1).

Growth properties and chromosome analysis: The growth curve of GEK cells showed that the exponential phase began at 3 days after inoculation. The GEK cells entered stationary phase 6 days after inoculation, then began to degenerate. When cultivated in DMEM medium supplemented with 10% FBS, the GEK cells can proliferated with a doubling time of about 49.5 h. Nearly 87% of the GEK cells had a chromosome number of 60, suggesting that the modal chromosome number of the established cell line was 60, which was coincident with chromosome number of goat (Fontanesi *et al.* 2010). These results indicated that the GEK cells are capable of stable growth *in vitro*.

Tumorigenesis assay: Tumorigenesis assay showed that solid tumors were found in all 10 BALB/c nude mice 28 days after inoculated with Hela cells. However, no solid tumor was found in all the BALB/c nude mice inoculated with GEK cells. This indicated that the GEK cells had no tumorigenic potency, suggesting that the GEK cell line established here could be used safely for vaccines manufacture.

Determination of marker protein expression: Immunocytochemistry assay showed that cytoplasm of GEK cells were stained to be brown (positive result). Cells served as negative control showed a color of light blue (Fig. 2). These results revealed that GEK cells were capable to express Vimentin and CK-18, which were considered as marker proteins of renal epithelial cells (Menko *et al.* 2014), suggesting that the established cell line had properties of renal cells, not vascular endothelial cells.

Analysis of influenza virus receptors on GEK cells: Immunofluorescent assay demonstrated that GEK cell contained α 2,3-SA receptor and α 2,6-SA receptor. Under the fluorescent microscope, GEK cells showed a stronger fluorescence than that of the MDCK cells, especially the GEK cells for SNA receptor test (Fig. 3), indicating that GEK cells expressed more influenza virus receptors than that of MDCK cells. Virus receptor emerged on the target cells, this is thought to be one of the key factors enabling influenza viruses replication in cells (de Graaf and Fouchier 2014, Tzarum *et al.* 2015). Considering that the GEK cell has more receptors, it may be more sensitive to influenza virus than MDCK cell.

Virus infection assay: Results indicated that both the GEK cells and MDCK cells showed high susceptibilities to influenza virus infection in the assay. GEK cells showed evident CPE at about 24 h after infection, which was obviously earlier than that of MDCK cells (about 36 h post infection). The infected GEK cells became round and swollen. Uninfected cells showed no sign of CPE. More

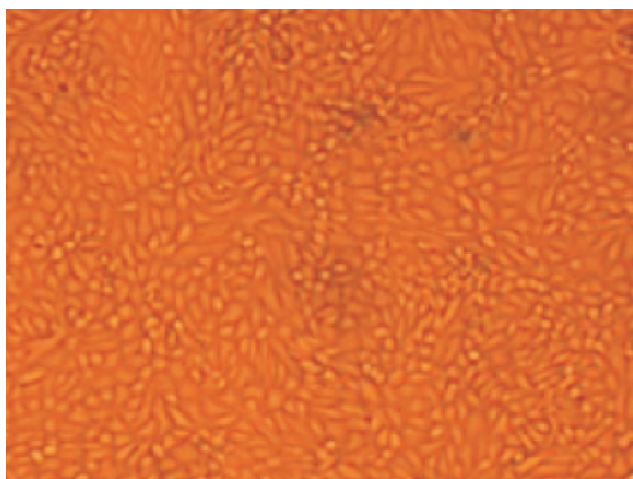


Fig. 1. Morphology of the monolayer formed by GEK cells (100 $\times$).

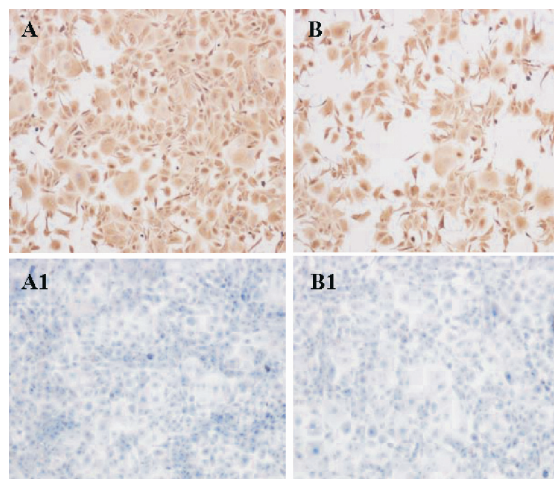


Fig. 2. (A,B,A1,B1). Analysis of marker proteins of GEK cell by immunohistochemistry (100 $\times$). **A**. Positive expression of vimentin; **B**. Positive expression of CK-18; **A1**. Negative control of vimentin; **B1**. Negative control of CK-18.

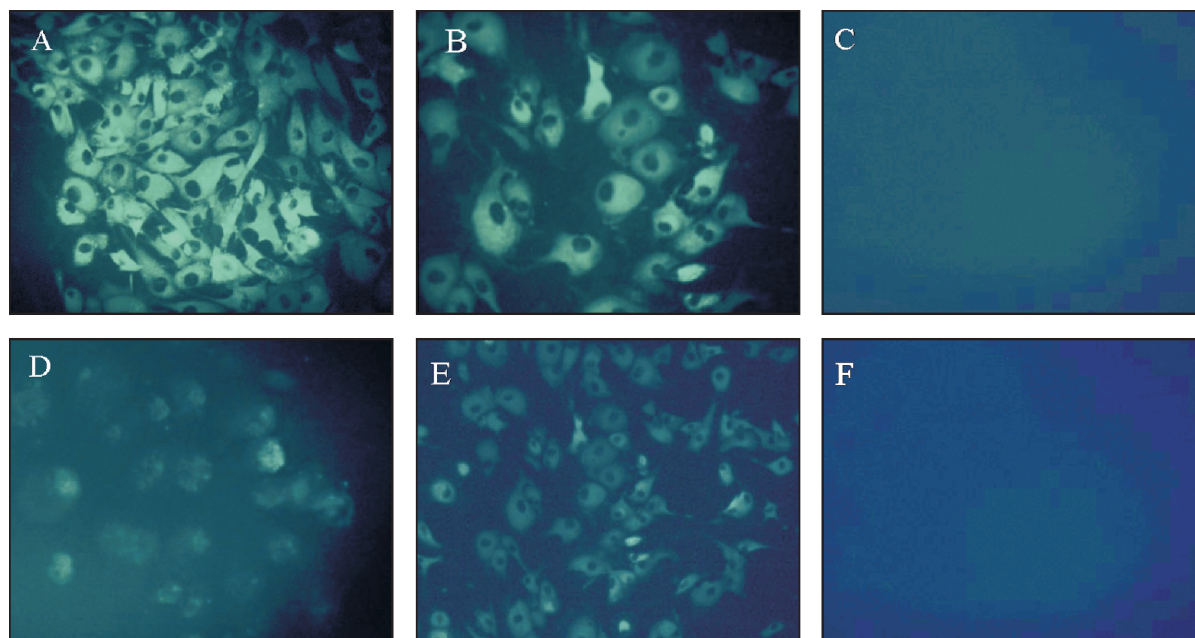


Fig. 3. (A-F). Comparison of MAA and SNA receptors of GEK cell and MDCK cell (400 $\times$). **A**. SNA receptor of GEK cell; **B**. MAA receptor of GEK cell; **C**. GEK cell control; **D**. SNA receptor of MDCK cell; **E**. MAA receptor of MDCK cell; **F**. MDCK cell control.

receptors might explain why cytopathic effect in GEK cells appeared earlier than that in MDCK cells. Earlier appearance of CPE could make people timely know if the cells are infected successfully. Hemagglutination assay also revealed that the HA titer of GEK cell reached pick at about 60h post-infection, and the maximum virus titer in GEK cells was comparable with that of MDCK cells (Fig. 4). MDCK is the most widely used cell line for the production of influenza vaccine today (Tapia *et al.* 2014). In our study, GEK cells had the same performance in producing virus as that of MDCK cells. High virus yield will contribute to the production of influenza vaccine.

All these suggested that GEK cell may be a promising candidate for influenza vaccine production. However,

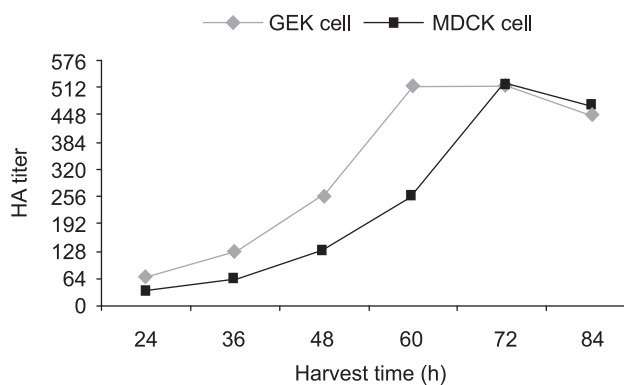


Fig. 4. Proliferation curve of influenza virus in GEK and MDCK cells. Data were mean values of 2 replicates.

whether GEK cell is susceptible to other serotypes of influenza viruses needs to be further investigated. The present study reports the development and characterization of the GEK cell line, which is susceptible for influenza virus propagation. Further investigation and application of the GEK cell line will contribute to influenza virus isolation, characterization and vaccine production.

ACKNOWLEDGMENT

We thank Dr Jinlin-Liu for his kind help in carefully editing the manuscript and our colleagues of Wuhan Institute of Animal and Veterinary Science for their technical assistance.

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