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RESEARCH ARTICLE

Establishment and evaluation of the goose embryo epithelial (GEE) cell line as a new model for propagation of avian viruses

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Abstract

In this study, we report the establishment and characterization of a new epithelial cell line, goose embryonated epithelial cell line (GEE), derived from embryonic goose tissue. The purified GEE cell line can efficiently grow over 65 passages in the M199 medium supplemented with 10% fetal bovine serum at 37˚C. Immunofluorescence assay was used to identify purified GEE cells as epithelial cell line by detecting expression of the Keratin-18 and -19. Further characterizations demonstrated that the GEE cell line can be continuously subcultured with (i) a high capacity to replicate for over 65 passages, (ii) a spontaneous epithelial-like morphology, (iii) constant chromosomal features and (iv) without an evidence of converting to tumorigenic cells either *in vitro* or *in vivo* study. Moreover, the GEE cell line can be effectively transfected with plasmids expressing reporter genes of different avian viruses, such as VP3, VP1 and F of goose parvo virus (GPV), duck hepatitis virus (DHV), and Newcastle disease virus (NDV), respectively. Finally, the established GEE cell line was evaluated for avian viruses infection susceptibility. Our results showed that the tested GPV, DHAV and NDV were capable to replicate in the new cell line with titers a comparatively higher to the ones detected in the traditional culture system. Accordingly, our established GEE cell line is apparently a suitable *in vitro* model for transgenic, and infection manipulation studies.

Introduction

Manufacturing technology is still based on the embryonated chicken eggs for propagation of avian viruses to produce vaccines against avian viral infectious diseases. However, the egg-based production system has some drawbacks, such as (i) specific pathogen-free (SPF) chicken

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eggs are expensive and sometimes it is difficult to continually keep SPF flocks completely free of pathogens, (ii) limitation of the manufacturing process of SPF-chicken eggs that may result in a drastic defect in the production process of vaccine doses, and (iii) process of virus propagation in embryonated eggs is usually time-consuming and labor intensive. Therefore, establishment of new flexible and scalable cell lines remains one of the major challenges of the avian vaccine industry. Avian cell-based production system provides a useful tool for virus propagation *in vitro* under certain conditions, and for virus production which is might be similar to circulating virus strains [1–3]. It allows producing high quantities of vaccines in short production cycles, therefore avoiding long processing production in embryonated eggs [4, 5]. Establishment and characterization of new cell lines might also provide an alternative tool to study (i) mechanism of viral pathogenesis, and (ii) immunological responses and associated gene expression in the field of host-virus interactions that will be subsequently essential for vaccine development. Development of new fibroblast cell lines that support isolation and propagation of avian viruses, such as goose parvo virus (GPV), duck hepatitis virus (DHV), and Newcastle disease virus (NDV) have already been characterized previously [6–10]. However, fibroblast cells show characteristic morphological changes of senescence after a few passages of the established cell lines. In an attempt to develop a continuous culture from embryonated chicken eggs, several difficulties have been reported during establishment and development such of these cell lines [11–13]. Indeed, our laboratory succeeded to establish an epithelial cell line from duck embryo tissue that can be (i) passaged for more than 65 times without any effects on their morphological and biological characteristics, and (ii) supported propagation of the DHAV with a titer comparatively similar to the titer of propagated virus in the embryonated egg [14].

In the present study, we focus on the development and characterization of goose embryo epithelial (GEE) cell line that could be cultured and passaged to establish a normal non-transformed epithelial cell line and offer more pliability for study biological properties and propagation of different avian viruses. We, therefore, developed and characterized an epithelial cell line from the primary tissue culture of embryonated goose and report that the established GEE cells can be efficiently retained their epithelial properties even after 65 passages. Growth, proliferation and chromosomal features of the established GEE cell line are detected also in this study. Moreover, Susceptibility of the GEE cell line for exogenous genes transfection and GPV, DHAV, NDV infection is determined.

Materials and methods

Animal ethics

Animal care procedures were performed in accordance with animal ethics guidelines and approved protocols. All animal experiments were approved by the Animal Ethics Shandong Lvdu Biotechnology Co., Ltd., Binzhou, Shandong, China. The Animal Ethics Committee approval number was SYXK 20160008.

Isolation and culture conditions of the cells

Eleven-day-old goose embryo eggs were purchased from a farm in Binzhou, China (Binzhou Kangyue Poultry Co., Ltd., China). Laying geese are periodically screened by the farm to monitor whether geese flocks are specific pathogen-free (SPF). After disinfection of the embryonated eggs, primary tissues were collected, dissociated mechanically and treated enzymatically exactly as described before [14] to yield the goose embryonic epithelial (GEE) cell line. In brief, internal organs of goose embryo eggs were excised, placed in a beaker containing phosphate buffer saline 1X (PBS-1X), and were washed three times using fresh PBS. Tissues were cut into

small pieces less than 0.5–1.5 mm3, transferred to a petri dish and incubated at 37˚C and 5% CO_2. At 5–7 days post incubation, isolated cells were then digested using 0.1% collagenase type I (Invitrogen) for 3–5 min. The single-cell suspension was obtained by sieving digested cells using a 40 μm cell strainer (Falcon, Corning). The obtained cell suspension was washed three times in PBS-1X, and then was converted to a new petri dish and incubated at 37˚C and 5% CO_2 for 5 days. The newly growing cells were digested again with a collagenase type I 0.1% and incubated for 7 days. The digestion steps with collagenase were repeated 4 times until a 100% of the purified GEE cell line was obtained. Sterile cloning cylinders were used in order to choose cells which have an epithelial appearance. A 200 μl of collagenase was placed inside the cylinder and treated cultures were then incubated at 37˚C until cells were detached from the plastic dish. Thereafter, the cell suspension was placed immediately into a 6-well plate (Corning).

Growth curve and population doubling time

To evaluate growth properties of the established GEE cell line, cells at passage 20, 45 and 65 were plated at a density of 5 x10^4 cells per well (24-well culture plate). The cells were counted using countess™ automated cell counter, and stained using trypan blue (Invitrogen) daily. The experiment was performed in three independent replicates, and lasted for 7 days. The population doubling time (PDT) was calculated with the following formula: TDP = t x log (2) / log (Nf/Ni), TD and t represents cell doubling time and duration of cell culture, respectively. While Nf represents the number of harvested cells and Ni represents the number of seeded cells. TD = t (lg2 / lgNt—lgN0) (TD and t represent cell doubling time and duration of cell culture, respectively. While, N_0 represent cell number after inoculation and N_1 represent cell number after culturing for t hours [15]. The Ni was 2000 in each passage and NF was calculated based on number of the harvested cells at 7th day of each passage.

Flow cytometry analysis of the cell cycle

Cell cycle analysis was carried out using flow cytometry as described before [16, 17]. Briefly, the established GEE cell line was trypsinized, suspended in PBS containing 2% FBS and 0.01% sodium azide and centrifuged at 1200 rpm. The cell pellet was fixed in 70% ethanol and kept at 4˚C for 30 min. The cells were treated with RNAse (5 mg/ml) for 30 min at 37˚C. After three times washing with PBS, the cells were stained with Propidium Iodide (PI) with a final concentration of 25 mg/ml in 1% sodium citrate at 4˚C for 15 min. Cell cycle analysis was carried out using FACSCalibur™ flow cytometer (BD FACSAria™ II), and the intensity of fluorescence was analyzed using ModFit LTTM software.

Karyotype analysis and tumorigenicity studies

Karyotype analysis of the established GEE cell line was performed according to the method that reported previously [14, 18]. Chromosomes were prepared with the established GEE cell line of 60^{th} to 65^{th} passages. The cells were exposed to 10 μM colcemid (Invitrogen) in fresh medium and incubated at 37˚C. After 2 h, the cells were trypsinized and collected by centrifugation at 1000 rpm for 10 min. The cells were fixed in methanol and glacial acetic acid, and spread on glass slides. Slides were stained with 3% Giemsa solution in PBS for 30 min at RT. Phase-contrast microscopy was used to count a total 100 randomly selected metaphase spreads.

To determine *in vitro* tumorigenic properties of the established GEE cell line, soft agar assay was performed as previously described [16].The SP2/0 mouse myeloma (SP2/0) cell line was used as a positive control (a gift from institute of biochemistry and cell biology, Shanghai,

China). The coated cells were incubated at 37˚C and 5% CO_2 for 3 weeks. Colonies formation were analyzed after 3 weeks and inspected under an OLYMPUS CKX_{41} microscope (Life sciences). Colonies were considered positive when they have 10 or more viable cells.

For *in vivo* tumorigenic study, balb/c nude mice (Beijing HFK Bioscience CO., Ltd., China) were used. All mice in this study were housed in isolation room at Animal Center of the Fourth Military Medical University, China and handled with appropriate ethics. Four- to five-weeks-old mice were randomly selected, and divided into two groups, with 3 mice in each group. Mice in both groups were injected subcutaneously (SQ) with either 0.2 ml of the established DEE or SP2/0 (positive control) cell line with a final concentration of 5×10^6 cells/ml. Animals in both groups were observed for tumor formation twice per week up to two months.

Transmission electron microscopy (TEM)

The established GEE cell line was examined with TEM as reported previously [19]. The GEE cell line at passage 65 were seeded in a T75 flask (Thermo Scientific) and allowed to proliferate. Proliferated cells were trypsinized, washed with 0.1 M PBS (pH 7.4), fixed in 2% glutaraldehyde in PBS for 30 min at RT, and then post-fixed in 2% osmic acid in PBS for 2 h at room temperature. The cells were dehydrated in a serial dilution of ethanol, and then embedded overnight in Epon-812 (Sigma). Specimens were stained with uranyl acetate and counter-stained with alkaline lead citrate. The JEM-2000EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) was used to examine the ultrastructure of the established GEE cell line.

Plasmid construction and transfection

To generate pcHA_GPV-VP3, the VP3 gene (1605 bp) of GPV was amplified by PCR using primers P1 (ATTGGATCCTGAAATGGCAGAGGGAGGA) and P2 (CGCTCGAGCGCCAG GAAGTGCTTATTTGA). Then, PCR products were digested with *BamHI* and *XhoI* and inserted into pcDNA_HA (pcHA; kindly provided by Dr. Suresh Tikoo, Saskatchewan University, Canada) resulting in pcHA_GPV-VP3. Similarly, to insert VP1 gene (720 bp) of DHAV and F gene (1662 bp) of NDV in pcHA, the same strategy was followed exactly as mentioned above using the primers P3 (ATAAAGCTTATGGGTGATTCTAACCAGTTGG), P4 (CGGGGATCC CTATTCAATTTCCAGATTGAG), P5 (TCCAAGCTTATGGGCTCCAAATCTTCTACCAGATC) and P6 (GCTCTAGACTCAGATTCTTGTAGTGGCTCTCCTTTG), respectively. PCR products of DHAV-VP1 and NDV-F were digested with (*HindIII* & *BamHI*), and (*HindIII & XbaI*), respectively, and then inserted into pcDNA_HA.

Transfection was performed as previously described [20]. Our established GEE cell line was cultivated on a coverslip in 6-well plate at 37˚C and 5% CO2. The cells were washed with Opti-MEM® (Life Technologies) and transfected with respective plasmid DNA (1 μg/well) following manufacturer's instructions of Lipofectamine™ 2000 (Life Technologies). After 6 h of incubation, the transfected reagents were removed, and replaced with fresh medium and incubated at 37˚C and 5% CO_2 for 24–48 h for further analysis.

Indirect immunofluorescence assay (IFA)

To evaluate the cytokeratin expression in the established GEE cell line, IFA was performed as described before [20]. The established GEE cell line was plated on a coverslip in 6-well plate at 37˚C and 5% CO_2 for 24 h. Duck embryo epithelial (DEE) [14] and chicken embryo fibroblast cells (DF-1) were used as a positive and a negative control, respectively. The cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature (RT), and permeabilized with 1% Triton X-100 in PBS for 2 min. After three times washing with PBS, fixed cell were incubated in the blocking buffer (PBS; pH 7.4 and 0.5% FBS) for 1 h

at RT. In order to determine expression of the Keratin-18 and -19, cells were incubated either with mouse monoclonal anti-keratin 18 antibody or mouse monoclonal anti-keratin 19 antibody (Wuhan Boster Biotechnology Co., Ltd., China). The corresponding anti-mouse secondary antibody labelled with Alexa Fluor® 488 (Green staining; Santa Cruz Biotech) was used.

To detect expression of GPV_VP3, DHAV_VP1 and NDV_F proteins, the HA (hemagglutinin epitope) amino acid tag sequence was fused to C-terminus of respective proteins, respectively. Then, monoclonal anti-HA antibody was used as primary antibody to detect expression of the target proteins. To further confirm expression of these proteins, collected hyperimmune serum from injected chicken with respective viruses was used as a specific primary antibody and anti-chicken IgY (IgG) (whole molecule)—FITC antibody produced in rabbit (Sigma) was used as a secondary antibody. After three times washing with PBS, cells were examined under OLYMPUS CKX_{41} inverted microscope (Life science), which supplied with trinocular head options and fluorescence upgrade capability to be suitable for imaging of GFP and other fluorescence applications.

Adaptation, titration and growth kinetics of viruses

The stock viruses of GPV (isolated from goose embryo from Binzhou, China), DHAV-1 SDE vaccine strain (provided by Quingdao Baoyite Biological Pharmaceutical Co., Ltd., China), and NDV lasota strain (purchased from institute of veterinary drug control, China), were used to infect the established cell line at a multiplicity of infection (moi) of 1. After incubation of virus-infected cells for 72 h, cells were subjected to 3-times freeze-thaw, then medium-containing virus was clarified by centrifugation. For viruses adaptation, established GEE cell line was reinfected with respective viruses for 10 times. To determine titer of respective viruses, the supernatant fluid of the adapted viruses was collected and tenfold dilutions were prepared in the M199 medium supplemented with 2% FBS. The cultured GEE cell line was infected with the respective viruses and then were incubated at 37˚C and 5% CO2. Titers of viruses were determined as described before [21, 22] using the 50% tissue infectious dose ($TCID_{50}$) assay on the established GEE cell line. Moreover, the 50% egg lethal dose (ELD_{50}) assay was used to measure titer of GPV using 9-day-old embryonated goose eggs (Binzhou Kangyue Poultry Co., Ltd., China), and NDV and DHAV using 9-day-old embryonated chicken eggs (Jinan Sipafasi Poultry Co., Ltd., China). To determine growth kinetics of GPV, DHAV, and NDV on the established GEE cell line, GEE cells were infected with respective viruses at a moi of 0.5. The infected cells were harvested at the indicated time points (12, 24, 36, 48, 72, 96, 108, and 120 hour post infection (hpi)) and analyzed using $TCID_{50}$ assay as early described.

Quantitative reverse transcriptase-polymerase chain reaction (qPCR)

In order to assess virus growth kinetics on the established GEE cell line, the established cells were infected with either GPV, DHAV or ND virus at a moi of 0.1. The infected cells were harvested at the indicated time points (12, 24, 36, 48, 72, 96, 108, and 120 hpi) and analyzed using $TCID_{50}$ assay as mentioned above and qPCR. The qPCR was done as described earlier [7]. Briefly, RNA was extracted at different time points (12, 24, 36, 48, 72, 96, 108, and 120 hpi) using RNA Extraction kit (Bioteke). The general RNA was reverse transcribed into cDNA using Thermo M-MLV transcriptase kit (Bioteke). Specific oligonucleotide primers (Table 1) were designed based on reference sequences of the VP3 gene of GPV (GenBank accession no. KC996729.1), VP1 gene of DHAV (GenBank accession no. DQ864514.3) and F gene of NDV (GenBank accession no. KY549653.1). Primers was used to amplify a 164, 230 and 176 bp of GPV_VP3, DHAV_VP1, and NDV_F, respectively. The amplified cDNA fragments of each gene were cloned separately into a clone vector, pMDT-18 (pM). The recombinant plasmids

Table 1. Oligonucleotides primers used for qPCR.

GenBank number	Primer name	Primers sequence	Length (base pairs)	Positions	Target gene
KC996729.1	GPV-F	5-CAGTCTGTGTCTCCAGCCAG	164bp	2542–2561	VP3
	GPV-R	5-TGTTGGTCGTAGGCCTTGTC		2686–2705	
DQ864514.3	DHAV-F	5-TCTGTCCAGGCACATGCACAT	230bp	468–488	VP1
	DHAV-R	5-CTCCACCTTTTCTGCACAAGA		677–697	
KY549653.1	NDV-F	5-TCGGTAGCGGCTTAATCACC	176bp	791–810	F
	NDV-R	5-CACTTTTGGGACAAGTGCCG		947–966	

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(pM_VP3, _VP1, and _F) were purified and used as standards for qPCR. The qPCR was performed by using LightCycler® 480II Real-time PCR system (Roche) in a total volume of 20 μl. The PCR conditions consisted of one cycle of 1 min at 95˚C followed by 40 two-step cycles of 20 sec at 95˚C and 20 sec at 61˚C. The analysis of PCR results were performed using LightCycler® 480II system software (Roche). The experiment was performed independently in three times and each sample of each experiment was also tested in duplicate.

Results

Establishment of the GEE cell line

All eggs batches used in this study to generate the GEE cell line were screened by an enzyme-linked immunosorbent assay to determine the presence of antibodies against avian viruses, especially GPV, NDV and DHAV (data not shown). The GEE cell line was established from primary tissues of the goose embryo. Within the first week, primary cultures were adhered to the surface of the cell culture petri dish (Fig 1A) and gradually formed epithelial cell colonies after several digestions with collagenase type I (Fig 1B). After serial passages, the number of epithelial cells gradually increased. Then, all of the formed epithelial cells adhered tightly to the bottom of the cell culture flasks in a monolayer, and later were characterized as epithelial cell

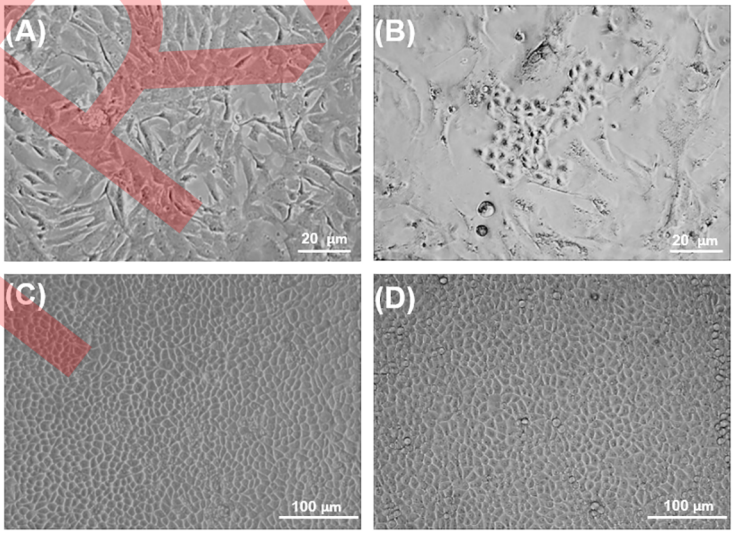

Fig 1. Morphology of the DEE cell line at different stages of the culture process. (A) Initial culture of the goose embryo tissue. (B) After several treatment of primary culture with collagenase, tightly packed epithelial-like cell colonies were observed. (C) Existence of cells with epithelial-like morphology after serial passages. (D) After freezing and thawing, the DEE cell line displayed a uniform shape of the epithelial cells. Figs 1A and 1C are excluded from this article's CC-BY license. See the accompanying retraction notice for more information.

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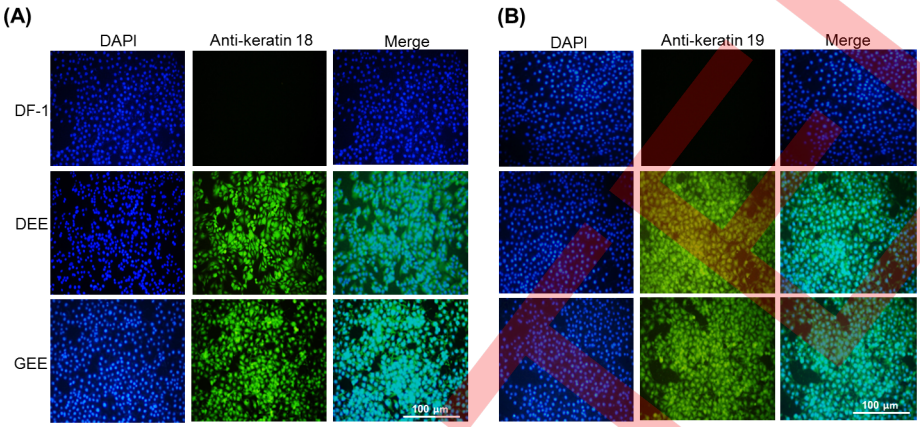

Fig 2. Expression of epithelial cell markers (keratin-18 and -19) in the established GEE cell line using IFA. Chicken fibroblast (DF-1) and Duck embryo epithelial (DEE) cells were used as a negative and a positive control, respectively. All of the respective cells were fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Then, cells were stained separately either with mouse monoclonal anti-keratin 18 (panel; A) or 19 (panel; B) antibody specific to epithelial cells. Antibody binding was revealed with Alexa Fluor 488 goat anti-mouse IgG secondary antibody. Nuclei were stained with DAPI (Vector Laboratories).

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line (Fig 1C). The frozen GEE cells at passage 65 were tested for retaining of their epithelial morphological characteristics. Our results showed that the recovered GEE cell line from liquid nitrogen was still maintained on the cuboidal morphology of the epithelial cells and other morphological properties of the epithelial cells (Fig 1D).

Epithelial marker expression in the GEE cell line

In order to detect expression of the epithelial marker, Keratin-18 or -19 in the established GEE cell line, IFA was carried out in presence of the DEE and DF-1 cell lines as a positive and a negative control, respectively. Mouse monoclonal antibody that targeting either the epithelial marker, keratin-18 or -19 was used as a primary antibody. While Alexa Fluor 488 goat anti-mouse IgG was used as a secondary antibody. As expected, our results demonstrated that both of the anti-keratin-18 and -19 antibodies were able to react with either established GEE or DEE cell line as a control positive but not with the DF-1 chicken fibroblast cell line (Fig 2). These findings are strongly proved that the established GEE cells are epithelial cells.

Ultrastructural characterization of the GEE cell line

Transmission electronic microscopy (TEM) was used to further observe ultrastructural of the established GEE cell line. The GEE cell line assessed by TEM were small round or oval shaped with oval nuclei (Fig 3A). The nucleus of these cells was large and irregular, showing one large prominent nucleoli (Fig 3B). Further analysis of the GEE cell line with TEM showed a high microvilli density on the cell surface (Fig 3B). Cytoplasm had a considerable quantity of secretory vesicles, lipid droplets (Fig 3C) and endoplasmic reticulum (Fig 3D). These results suggest that the established GEE cells maintained their epithelial characteristics even after several passages of the cells.

Growth characteristics of the GEE cell line

To assess growth properties of the established GEE cell line, the growth curve was carried out for established cells at passage 20, 45 and 65. Results of the growth rate capability of the GEE

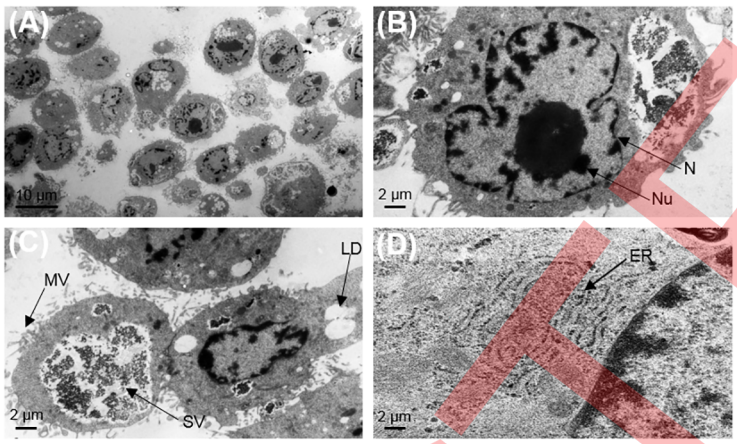

Fig 3. Ultrastructural characteristics of the GEE cell line using TEM. (A and B) Morphology of the cell line was cuboidal and nucleus (N) was large and irregular containing a large prominent nucleolus (Nu). (C and D) Cell surface was covered by abundant microvilli (MV) of irregular shape. Cytoplasm had a significant quantity of secretory vesicles (SV), lipid droplets (LD) and endoplasmic reticulum (ER). Fig 3D is excluded from this article's CC-BY license. See the accompanying retraction notice for more information.

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cell line at all different passages showed a rapid growth rate at 3–5 day post culture and reached a maximal growth rate near to the 6th day, followed by a reduction in growth rate of the GEE cell line by the 8th day without any significant changes between different passages of the GEE cell line. (Fig 4A). Furthermore, population doubling time (PDT) of the established GEE cell line of each passage was calculated based on the above mentioned formula. Our findings revealed that PDT of passage 20, 45 and 65 of the GEE cell line was 16.3, 18.12, 18.98 h, respectively without any significant changes between different passages of the established cell line (Fig 4A). On the other hand, cell cycle of the established GEE cell line was determined using flow cytometry. The PI as a fluorescent dye was used to stain cellular DNA of the established

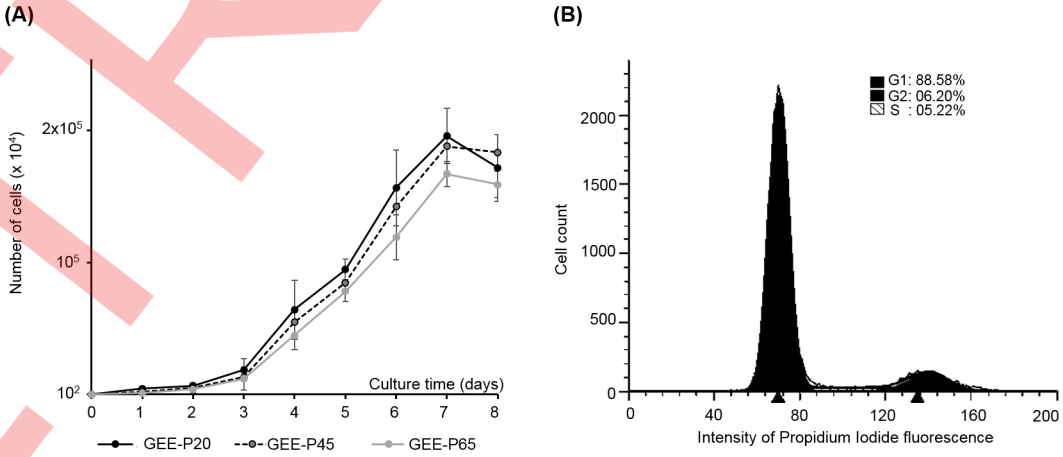

Fig 4. Growth properties of the GEE cell line. (A) Cellular growth curve of the GEE cell line. Cells at passages 20, 45 and 65 were grown at 37˚C in a 6-well plate containing 2 ml of the M199 medium supplemented with 10% fetal bovine serum until they reach confluency and then were collected at indicated time points for counting. Errors bars represent standard deviations from three independent experiments. (B) Flow cytometry was used to analyze the cell cycle of the GEE cell line. Cellular DNA was stained with the Propidium Iodide fluorescent dye and fluorescence intensity of cells was measured in the G1, S and G2 phases of the cell cycle.

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GEE cell line. The quantity of the DNA in each cell line was correlated to the fluorescence intensity of stained cells at certain wavelengths. Our results demonstrated that the fluorescence intensity in stained GEE cells was 88.58%, 5.22%, and 6.20% in Gap 1 (G1), synthesis (S) and Gap 2 (G2) phases of cell cycle proliferation, respectively (Fig 4B). We concluded from our findings that most of the GEE cell accumulated in the Gap 1 phase, suggesting that the established GEE cell line is characterized by a fast growth rate and a great proliferative activity.

Chromosomal and tumorigenic analysis

In order to determine chromosomal structure of the established GEE cell line, chromosomal analysis was conducted twice by randomly selecting the field of vision of the established GEE cell line at passage 65. Representative metaphase spread and karyotype of the GEE cell line demonstrated 39 pairs of chromosomes specific to goose (2n = 78) (Fig 5A). We concluded from these results that the GEE cell line had a normal diploid configuration with a chromosomal number similar to avian origin cells [23, 24], and can be normally propagated without any chromosomal abnormalities during its division.

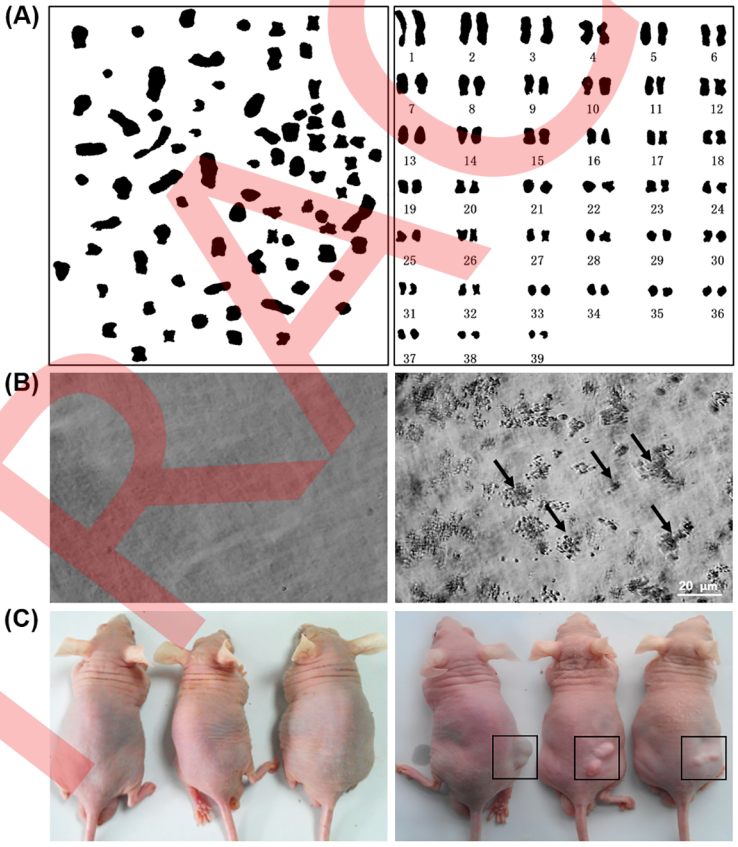

Fig 5. Chromosomal analysis and tumorigenicity study of the GEE cell line. (A) For chromosomal analysis, representative metaphase spread and karyotype of the GEE cell line showed 39 pairs of chromosomes specific to goose (2n = 78). For tumorigenicity study, (B) soft agar assay was used to analyze transformation properties of the GEE cell line *in vitro*. Inverted microscope (OLYMPUS CKX41) was used to image colonies formation either in GEE cell line (left panel) or in SP2/0 cell line (right panel). Colonies formation in the SP2/0 cell line referred with arrows. (C) The balb/c nude mice were inoculated SQ with either GEE (left panel) or SP2/0 cell line (right panel) for *in vivo* study. Tumor formation in the injected mice with SP2/0 indicated with squares. The left panel in Fig 5C is excluded from this article's CC-BY license. See the accompanying retraction notice for more information.

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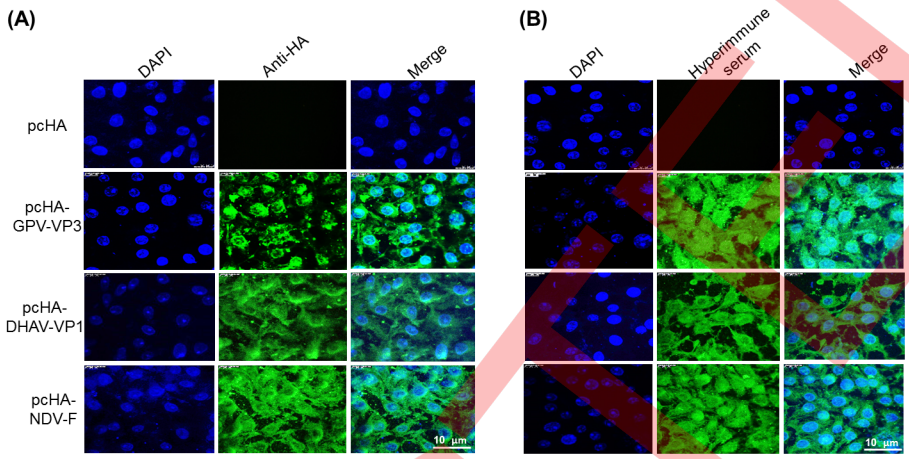

Fig 6. Analysis of exogenous genes expression in the GEE cell line by IFA. The GEE cell line was mock-transfected with pcHA or transfected with either pcHA_GPV-VP3, pcHA_DHAV-VP1 or pcHA_NDV-F. At 48 post transfection, cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Monoclonal anti-HA antibody and hyperimmune serum from injected chicken with respective viruses were used as a primary antibody in (panel; A) and (panel; B), respectively. While Alexa Fluor® 488 (Green staining; Santa Cruz Biotech) and anti-chicken IgY (IgG) (whole molecule)—FITC antibody produced in rabbit were used as secondary antibodies. Nuclei were stained with DAPI (Vector Laboratories).

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Tumorigenicity study of the established GEE cell line was determined either *in vitro* or *in vivo*. Soft agar assay was performed to analyze whether the GEE cells can be transformed to tumorigenic cells *in vitro*. Results showed that the established GEE cell line did not show any colonies compared to SP2/0 cell line, which showed different colonies on the soft agar (Fig 5B). Furthermore, balb/c nude mice were injected SQ with either established GEE cell line or SP2/0 cell line as a positive control to determine tumorigenicity properties of the GEE cell line *in vivo*. Our results demonstrated that tumors formation not observed in the inoculated mice with the established GEE cells (Fig 5C, left panel), but observed in the inoculated mice with SP2/0 cells (Fig 5C, right panel). Taken together, we concluded that the established GEE cell line can be propagated normally without transformation to tumorigenic cells.

Cell susceptibility for exogenous genes transfection

In order to evaluate susceptibility of the established GEE cell line for exogenous genes transfection, IFA was used. GEE cells at passage 65 were mock-transfected with pcHA or transfected with either pcHA_GPV-VP3, pcHA_DHAV_VP1 or pcHA_NDV_F. At 48 h after transfection, cells were observed for transfection efficiency using either anti-HA or specific antibody against each protein (Fig 6A and 6B). The IFA results showed that the GEE cell line can be efficiently expressed either GPV-VP3, DHAV_VP1 or NDV_F. We concluded from our findings that the GEE cell line is a suitable as an *in vitro* model for transfection and gene expression studies.

Viral susceptibility

The susceptibility of the GEE cell line either to GPV, DHAV or NDV virus was determined by evaluating formation of the virus-induced cytopathic effects (CPEs) and the 50% tissue culture infective dose ($TCID_{50}$). Furthermore, qPCR was also used to monitor whether the new established GEE cell line can be used to support avian viruses replication. Firstly, adaptation of the

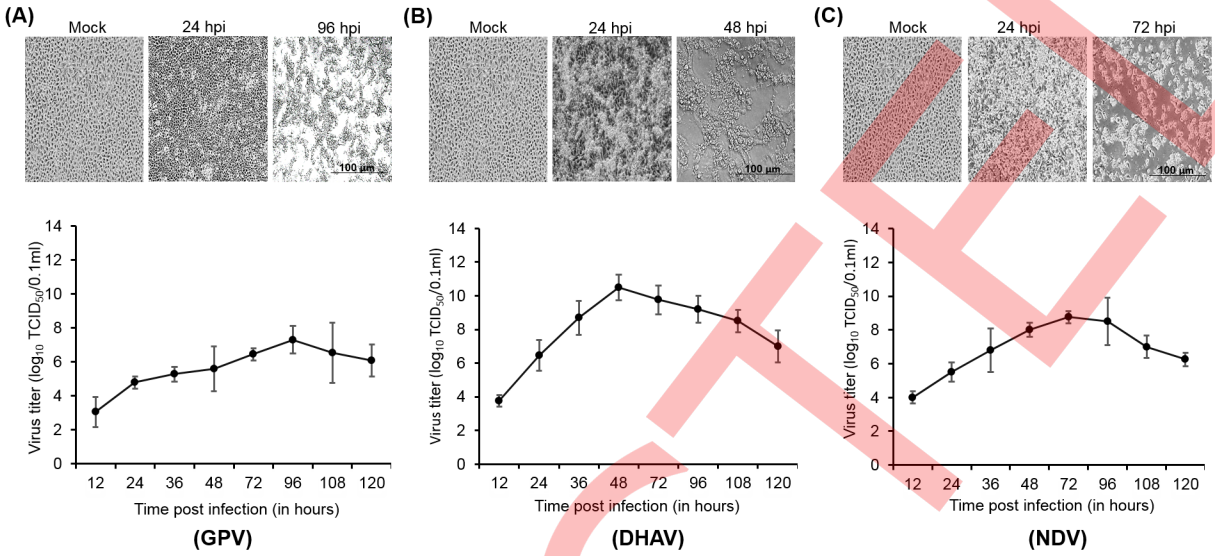

Fig 7. Susceptibility of the GEE cell line to GPV, DHAV and NDV infection. The CPEs following GPV, DHAV and NDV infection of the GEE cell line in the presence of uninfected cells as a mock were evaluated (A, B, and C; upper panel, respectively). Extensive CPEs associated with a rounding of the cells and destruction of the monolayer was observed at 96, 48, and 72 hpi with GPV, DHAV and NDV, respectively. Kinetics analysis of GPV-, DHAV- and NDV-infected GEE cells (A, B, and C; lower panel, respectively). Supernatant from infected cells with respective viruses was collected at diffident time points and titers of each virus were determined using $TCID_{50}$ assay. The data are the Mean ± S. E. from three independent experiments. The Mock panels in Figs 7A-B and Fig 7C are excluded from this article's CC-BY license. See the accompanying retraction notice for more information.

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respective viruses to the 65th passage of GEE cell line were done by propagation of each virus for several times and then the CPEs of adapted viruses were evident in cultures of the established GEE cell line of 65 passages at 24 hpi (Fig 7A, 7B and 7C; upper panel). Thereafter, CPEs were started to spread locally in the infected GEE monolayers where cells became rounded. At 96, 48, and 72 hpi of the GPV, DHAV and NDV, respectively, entire monolayers of the GEE cell line were affected by CPEs and formed a network of aggregated cells, and their shape varied from flat to spheroidal (Fig 7A, 7B and 7C; upper panel). While no CPEs were observed in uninfected control cells (Fig 7A, 7B and 7C; upper panel). Viral culture supernatant of each respective virus was collected from infected-GEE cells and used to determine reinfection ability of each virus to infect normal GEE. Our results showed that culture supernatant of each virus was able to re-infect normal GEE cells (data not shown). Viral replication efficiency was tested in the established GEE cell line and viral titers of GPV, DHAV and NDV viruses, respectively, were determined at different time points (Fig 7A, 7B and 7C; lower panel). Our results showed that titers of GPV, DHAV and NDV viruses were increased dramatically at 96, 48, and 72 hpi, respectively, until reaching maximal level of $10^{7.5}$, $10^{10.5}$, and $10^{8.75}$ $TCID_{50}$/1 ml (Fig 7A, 7B and 7C; lower panel). Further analysis was used to determine the ELD_{50} of propagated viruses in the GEE cell line compared to ELD_{50} of viruses that propagated only in embryonated eggs. Our findings showed that the ELD_{50} of propagated GPV, DHAV and NDV in the established cell line was $10^{5.8}$, $10^{10.2}$, and $10^{8.5}$ ELD_{50}/1 ml, while ELD_{50} per 1 ml of propagated GPV in goose embryo was $10^{4.75}$, and propagated DHAV and NDV in chicken embryo was $10^{10.2}$, and $10^{8.5}$, respectively. To further demonstrate that the GEE cell line can be supported avian viruses replication, the copy number of viral genomes was determined by qPCR (Fig 8). For qPCR, established cell line was infected at different time points with either GPV, DHAV or NDV. Subsequently, total cellular RNA from infected cell line was prepared and reverse transcribed into cDNA. Standard curves were developed based

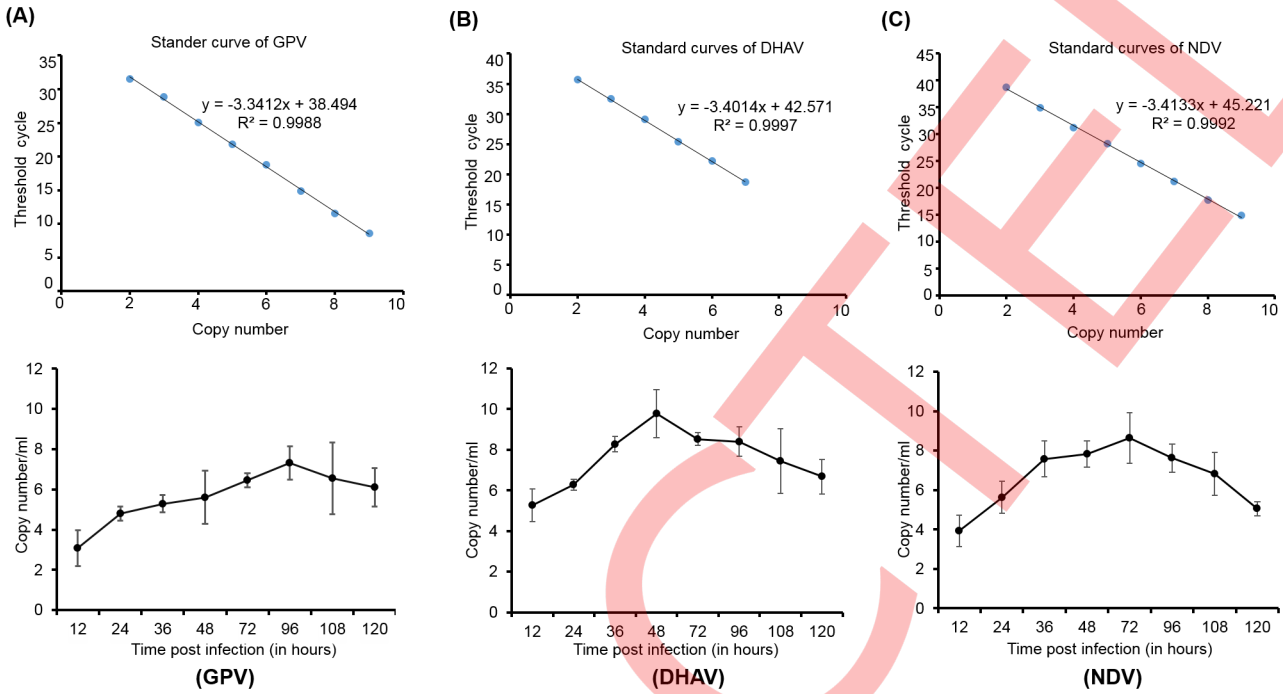

Fig 8. Standard curves of the qPCR and replication kinetics of the propagated viruses in the GEE cells. (A, B, and C; upper panel) For stander curves of the qPCR, the purified recombinant plasmids DNA of pC_VP3, _VP1, and F were used as standard DNA templets. The threshold cycle was plotted against the copy number of the standard DNA templets. Y represents the threshold cycle and R^2 represents correlation coefficient. Growth kinetics of GPV, DHAV and NDV viruses in the GEE cell line (A, B, and C; lower panel, respectively). The GEE cell line was infected with indicated viruses at a moi of 0.1 and cellular RNA were collected at different time points (12, 24, 36, 48, 72, 96, 108, and 120 hpi) and then reverse transcribed into cDNA. The viral copy numbers were measured by qPCR. All samples were performed in three independent times and errors bars represent standard deviations.

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on the purified recombinant plasmids DNA of pM_VP3, _VP1 and _F and the linearity and efficiency of the qPCR reaction appeared in the Fig 8A, 8B and 8C; upper panel. The copy number of viral nucleic acids, which isolated from GPV-, DHAV- or NDV-infected GEE cell line was reached to highest copy numbers of $10^{7.313}$, $10^{9.774}$, and $10^{8.633}$ copies/ml, at 96, 48 and 72 hpi., respectively (Fig 8A, 8B and 8C; lower panel). The results obtained by qPCR were correlated to the results of the viral growth kinetics experiment. Therefore, taken together, we concluded that the GEE cell line can be strongly supported the GPV, DHAV and NDV viruses infection with a high titer level compared to traditional method using embryonated egg.

Discussion

Although embryonated eggs are still used for isolation of avian viruses and development of new vaccines, cell cultures are the target choice for the most laboratories to reduce adverse features of embryonated eggs. The current available fibroblast cell line, embryonic goose fibroblast cell line CGBQ (ATCC® CCL-169™), has been used previously to study biological properties of the GPV *in vitro* (Qiu et al., 2005; Yin et al., 2012). However, fibroblast cell lines have disadvantages, such as (i) cell characteristics can be changed after a period of growth and may become a quite different from those found in the initial passages, (ii) has a limited life span and (iii) a high cost represented by maintaining a continuous supply. Consequently, therefore, the establishment of an epithelial cell line derived from embryonic goose tissue was

the focus of this study in order to develop a new cell line with a stander properties and a wider viral susceptibility. Heterogeneous populations of fibroblast- and epithelial -like cells were obtained from collagenase enzymatic digestion when cultured on a petri dish. Efficient disaggregation of epithelial cells was achieved by different digestion of heterogeneous populations of the cultured cell line with collagenase, which allows more efficient disaggregation of epithelial cells [14, 25]. Purified cell colonies with epithelial-like morphology were optimally cultured at 37˚C in M199 medium supplemented with 10% FBS until confluence. At the 65th passage, the established GEE cell line was successfully stored in liquid nitrogen. Then, recovery of the stored cells from cryopreservation was confirmed.

It was important to investigate epithelial characteristic features of the established cell line before continuing with further experiments, therefore, the immunofluorescence assay was used to detect expression of the cytokeratin-18 or -19 in the established GEE cell line. Cytokeratins are intermediate filament proteins and are one of the epithelial markers of epithelial cells, which can be used to investigate many aspects of epithelial cell biology [26, 27].

To the best of our knowledge, our study is the first study describing GEE cell line established from embryonic goose tissue, therefore, growth characteristic properties of the established cell line were analyzed in order to provide significant effects on the results of further experiments. Growth curve analysis of the established GEE cell line at at passage 20, 45 and 65 demonstrated a typical growth pattern with a population doubling time of about 16–18 h, and without any significant changes in growth properties of the established cell line. Similar findings have been observed for previously established cell lines from embryonated duck tissues [6, 14]. Flow cytometry was used to analyze cell cycle of the GEE cell line and our findings demonstrated an arrest not only in the G2 phase but also in the G1 (S) phase of cell cycle proliferations. These cell cycle arrests ensure that DNA damage can be avoided or repaired before the cell enters S phase, where the damaged DNA would be replicated [28, 29]. We currently conclude from our findings that the established GEE cell line can be continuously grown with a fast growth rate and a great proliferative activity *in vitro*.

Diploid chromosomal number of cell culture populations of the established GEE cell line was estimated to exhibit a similar a chromosomal number similar to the earlier report in avian origin cells [23, 24], implying that our established cell line can be proliferated normally without any abnormalities in its chromosomal number.

In order to address whether the established GEE cell line has to transform to tumor cells, the transforming activity of the established GEE compared to mouse myeloma SP2/0 cell line was determined by *in vitro* and *in vivo* tumorigenicity studies. Mouse myeloma SP2/0 cell line is one of the most frequently used parental cell lines in tumorigenicity studies [30]. Both *in vitro* and *in vivo* studies on the SP2/0 cell line demonstrated a transforming activity and a capacity to induce tumors in nude mice. Interestingly, while both tumorigenicity studies on the established GEE cell line did not exhibit any neoplastic transformation properties either *in vitro* or *in vivo study*.

Ultrastructure investigation of the established GEE cell line revealed typical epithelial characteristics as reported previously in different avian epithelial cells [19, 31]. The results obtained by TEM showed that cell surface of the established GE cell line was covered with a high density of microvilli, which leads to increase the surface area of cells and more efficient uptake of nutrients. The presence of a fairly well developed endoplasmic reticulum and high quantity of secretive vesicles and lipid droplets suggest that the established GEE cell line has a robust proliferative activity.

It is well known previously that the transfection efficiency of plasmid DNA is quite different among earlier used cell lines *in vitro* experimental research [32]. Exogenous genes expression in mammalian cell lines have a crucial role in the modern experimental biology, such as

expression of viral proteins one by one in the absence of other viral proteins has an importance to detect the role of each protein in the replication cycle [33]. Therefore, it was important in our study to evaluate whether the established GEE cell line is a suitable cell line for DNA transfection. The GEE cell line was transfected with DNA of pcHA plasmid harboring various avian viral genes, such as GPV-VP3, DHAV_VP1, and NDV_F. Our findings showed that the GEE cell line can be efficiently transfected with each plasmid under the effect of cytomegalovirus (CMV) promoter, implying that the established GEE cell line could serve as an *in vitro* system to determine the role of avian viral genes. Although the embryonic goose fibroblast cell line CGBQ (ATCC® CCL-169™) was reported previously as a transient expression system to determine the expression strategy of GPV *in vitro* (Qiu et al., 2005). However, the short lifespan of the CGBQ cells making the continuous GEE cell line is the transfection recipient of choice in the stable expression system.

One of the important goals of this study is to evaluate the susceptibility of the established GEE cell line for infection with different avian viruses, such as GPV, DHAV, and NDV. Therefore, observation of CPEs, virus titration, and presence of viral RNA, was determined for GPV, DHAV and NDV separately at different time points of the infection. Monitoring of viral-induced cytopathic effects of the propagated GPV, DHAV and NDV in the established GEE cell line, resulted in cytopathic effects visible at different time points post infection of each virus by low-power microscope. In addition, $TCID_{50}$ and ELD_{50} assay were used to determine kinetics of infective viral titers of each virus separately at different time points of the infection. It has been reported previously that the GPV can be propagated in goose embryo fibroblasts [9] and muscovy duck embryo fibroblast cell lines [7]. To relate our findings to previous studies, the established GEE cell line was evaluated for GPV-infection susceptibility and showed a strong susceptibility to GPV. The growth kinetics assay revealed a maximum viral titer of $10^{7.5}$ $TCID_{50}$/ml at 96 hpi. Interestingly, the ELD_{50} of the propagated GPV in the GEE cell line was relatively higher than the ELD_{50} of GPV, which propagated only in the embryonated eggs. Susceptibility of the established cell line to DHAV infection was detected and our results showed that the GEE cell line can efficiently support DHAV virus replication with a maximum virus titer of $10^{10.5}$ $TCID_{50}$/ml at 48 hpi and $10^{10.2}$ ELD_{50}/1 ml, which higher than the ELD_{50} of propagated DHAV in the embryonated eggs. We and others, have previously shown that Duck epithelial cell line (DEE) [14], Duck embryo [6] and Muscovy duck embryo fibroblast cell lines [7] have been used to facilitate isolation and propagation of the DHAV. While these studies have been demonstrated that the titer of the DHAV was a relatively low compared with the titer of the propagated DHAV in the GEE cell line. The established GEE cell line was also tested for the NDV infection and we found that the GEE cells can strongly support replication of the NDV as reported previously [8, 10]. While our established GEE cell line exhibited a strong susceptibility to NDV with a viral titer of $10^{8.75}$ TCID50/ml after 72 hpi and $10^{8.5}$ ELD_{50}/1 ml. In an attempt to test the DEE cell line established previously in our laboratory (Wang et al., 2016) whether it can support replication of other avian viruses such as NDV and GPV. We found that the DEE cell line was not a suitable model to support replication of those viruses *in vitro* (data not shown). In order to confirm susceptibility of the GEE cell line for GPV, DHAV and NDV, copy numbers of viral genome of each propagated virus in the established GEE cell line were investigated separately by qPCR to confirm obtained results by $TCID_{50}$ and ELD_{50} assay. The growth rate of propagated respective viruses (GPV, DHAV and NDV) in the GEE cell line was started to increase gradually until reached to highest rate at 96, 48 and 72 hpi., where the viral genome replication reached a $10^{7.313}$, $10^{9.774}$, and $10^{8.633}$ copies/ml, respectively. In agreement with a previous study [7] which also showed that the copy number of the propagated GPV and DHAV in muscovy duck fibroblast cell lines peaked with $2x10^6$ and 10^6, respectively. Our finding that the GEE cell line can be supported replication of the GPV was not surprising,

as previous data have shown that other cell lines can also be facilitated propagation of the GPV [7]. However, we were surprised by the ability of the established GEE cell line to assist replication of other avian viruses, such as DHAV and NDV with high infectivity titers compared to other cell lines [6, 8, 10, 14]. This indicated that the established GEE cell line is very sensitive to avian viral infection.

In conclusion, we succeeded in establishing and characterization of a novel goose embryonated epithelial cell line. Our findings support the assumption that this line could be used as a fundamental tool in the propagation of avian viruses and may be useful for the development of vaccines. Our further aim is to dissect the susceptibility of our established GEE cell line for infection with other important avian viruses compared to other cell lines.

Supporting information

S1 Checklist. Arrive checklist.
(DOCX)

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Validation: Abdelrahman Said.

Writing – original draft: Abdelrahman Said.

Writing – review & editing: Wenxiu Wang, Abdelrahman Said, Zhiqiang Shen.

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