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Characterization of a novel cell line from the caudal fin of koi carp *Cyprinus carpio*

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A continuous cell line (KF-101) derived from the caudal fin of the koi carp *Cyprinus carpio* was established and characterized. The KF-101 cell line multiplied abundantly in Leibovitz's L-15 medium containing 10% foetal bovine serum at 25° C, and was subcultured for >90 passages over a period of 3 years. Immunocytochemistry revealed that the KF-101 cells contain keratin, junction proteins connexin-43 and occludin, and ectodermal stem-cell marker Pax-6, but not vimentin. Furthermore, the KF-101 cells reacted with anti-human DARPP-32 and anti-human GATA-4 antibodies, and the labelling was regulated according to the cell cycle. The labels of the DARPP-32 and GATA-4 antibodies in the KF-101 cells were the suggested phosphatase-1 inhibitor-1 and GATA-3, respectively. In addition, the KF-101 cells were susceptible to koi herpesvirus but were resistant to eel herpesvirus, iridovirus, grouper nodavirus and chum salmon (*Oncorhynchus keta*) virus. The results indicate that the KF-101 cells are suitable materials for investigating biological and virological development.
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Key words: DARPP-32; epidermis; GATA; herpesvirus; Pax-6; PP1 inhibitor-1.

INTRODUCTION

Koi fish are an ornamental variety of domesticated common carp *Cyprinus carpio* L. 1758. It is a member of the family Cyprinidae, which includes numerous other fishes such as silver carp *Hypophthalmichthys molitrix* (Valenciennes 1844), crucian carp *Carassius carassius* (L. 1758) and grass carp *Ctenopharyngodon idellus* (Valenciennes 1844). *Cyprinus carpio* is one of the most economically valuable species in aquaculture worldwide, with 3 million tMt harvested annually; the species is especially valuable in Asia (http://www.fao.org/fishery/en/). Several continuous cell lines have been developed from normal and tumour *C. carpio* tissues. These cell lines include CaPi from the pituitary gland, CCB from the brain; CFC, KF-1, KFC and KCF-1 from the fin and EPC from the epithelioma papulosum. These cell lines have been used successfully to isolate and study fish viruses (Ribeiro & Ahne, 1983; Sano

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et al., 1985; Ronen *et al*., 2003; Dishon *et al*., 2005; Hedrick *et al*., 2005; Dong *et al*., 2011).

Fish cell lines have been developed from a broad range of tissues including those from the ovary, fin, swim bladder, heart, spleen, liver, eye, muscle, vertebrae, brain and skin (Lakra *et al*., 2011). Despite the potential use of these cell lines in the fields of carcinogenesis, developmental biology, physiology, toxicology and transgenics, the identity and molecular expression of the majority of these cell lines are inadequately characterized. The cell lines tend to be used in a limited capacity for virus isolation and propagation. These challenges may be overcome by combining cell cultures with immunocytochemistry and molecular techniques.

In this study, a new continuous cell line (KF-101) was established from the caudal fin of koi *C. carpio*. To clarify the identity of the KF-101 cells, the cells were examined for molecular expressions by specific antibodies and reverse-transcription polymerase chain reaction (RT-PCR) in addition to a growth curve. Also, the cells were examined for susceptibility to fish viruses such as koi herpesvirus, grouper nervous necrosis virus, iridovirus and aquareovirus.

MATERIALS AND METHODS

PRIMARY CULTURE AND SUBCULTURE

Fish, each weighing *c*. 20 g, were obtained from a local ornamental fish market in Taipei. For the primary culture, a fish was anaesthetized in iced water, and the body surface was wiped several times with an alcohol swab. The caudal fin was removed and washed thrice with phosphate-buffered saline (PBS) supplemented with antibiotics (1000 IU ml^{-1} penicillin and 1000 μg ml^{-1} streptomycin). After being washed, the tissue was minced with scissors and then transferred into a 25 cm^2 culture flask (Nunc; www.nuncbrand.com) containing Leibovitz's L-15 medium (Gibco; www.invitrogen.com) supplemented with 15% foetal bovine serum (FBS) (HyClone; www.biopharminternational.com) and antibiotics (100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). The flask was then incubated at 25° C. Each week during the experimental period, half of the growth medium was refreshed. When the primary cells reached confluence, the medium was removed and the cells were detached using a 0·1% trypsin-EDTA solution (Sigma; www.sigmaaldrich.com). The detached cells were resuspended in the growth medium at a split ratio of 1:2 and incubated at 25° C. After 10 passages, the concentration of FBS in the growth medium had decreased from 15 to 10%. The subcultures were stored in liquid nitrogen after every 10 passages. The freezing medium consisted of L-15 growth medium and 10% dimethyl sulphoxide (DMSO). The KF-101 cells at passages 60–85 were used in the experiments described as follows.

GROWTH CURVE ANALYSIS

The KF-101 cells at passages 60–70 were seeded in 25 cm^2 culture flasks and incubated at 25° C. The number of cells in duplicate flasks was counted daily for 5 days, using a handheld automated cell counter Scepter (Millipore; www.millipore.com). The experiment was repeated twice.

IMMUNOFLUORESCENCE ASSAY

The KF-101 cells were grown on circular glass cover slips in a four-well tissue culture plate at 25° C for 1–3 days and were then fixed in formaldehyde (3·7% in PBS, v/v) for 10 min at room temperature. The cells were permeated with 1% Triton X-100, washed several times in PBS and then incubated with primary antibodies at 37° C for 1 h. The primary

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TABLE I. Primary antibody, target, source and dilution for immunocytochemistry

Antibody	Target	Species and clone	Company	Dilution
A2B5	Oligodendrocyte progenitor cells	Mouse 105	Sigma	1:100
Brain lipid-binding protein	Radial glial cells	Rabbit polyclonal	Abcam	1:200
Connexin-43	Epithelial gap junctions	Rabbit polyclonal	Sigma	1:200
DARPP-32	Dopaminergic neurons	Rabbit polyclonal	Abcam	1:200
Galactocerebroside	Oligodendrocytes	Rabbit polyclonal	Chemicon	1:100
GATA-4	Mesendodermal cells	Rabbit polyclonal	Abcam	1:200
Glial fibril acidic protein	Astroglia	Mouse GA5	NeoMarkers	1:200
Keratin	Epithelia	Mouse C11	NeoMarkers	1:200
Occludin	Epithelial tight junctions	Rabbit polyclonal	National University of Kaohsiung	1:200
Olig2	Oligodendrocytes and some neurons	Rabbit polyclonal	Abcam	1:100
Pax-6	Ectodermal stem cells	Rabbit polyclonal	Abcam	1:200
Sox2	Stem cells	Rabbit polyclonal	Abcam	1:200
Tyrosine hydroxylase	Dopaminergic neurons	Mouse TH-2	Sigma	1:200
Vimentin	Mesenchymal cells	Mouse V9	NeoMarkers	1:200

antibodies used in this study are shown in Table I. The specificity of the antibodies on fish cells had previously been verified, apart from occludin (Wen *et al*., 2008*a*). The anti-occludin antibody was developed in the laboratory using recombinant grouper occludin as an immunogen; the specificity was confirmed by the immunoblotting process. After being washed several times, the cells were incubated with an appropriate secondary antibody at 37° C for 30 min. The secondary antibodies were Alexa Fluor 488-conjugated anti-mouse IgG (1:200) and Alexa Fluor 568-conjugated anti-rabbit IgG (1:200). The nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI; Sigma), which was applied for 1 min. The labelled cells were visualized under an Axiovert 200 fluorescence microscope (Carl Zeiss; http://corporate.zeiss.com/).

IMMUNOBLOTTING

The KF-101 cells (at passages 70–80) were rinsed thrice with PBS. The cells were then collected by scraping them into an extraction buffer containing 0·15 M NaCl, 50 mM Tris–HCl (pH 7·4), 2 mM EDTA, 1% sodium dodecyl sulphate (SDS) and protease inhibitor (Set I; Merck Ltd; www.merck.tw). The total protein content was measured using a protein assay solution (Bio-Rad; www.bio-rad.com). After dilution, 10 μg of protein was subjected to SDS-polyacrylamide gel electrophoresis (12% gel), and the protein bands were electrotransferred to a polyvinylidene difluoride membrane (Pall Corp.; www.pall.com). Membranes were blocked

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TABLE II. Primer sets used for reverse-transcription in this study

Primer set	Sequences (5′–3′)	Product size (bp)	Annealing (° C)	Reference
Bmp4	F: gtttaacctcagcagcatcc R: agccctccactaccatttcc	782	55	Wen *et al*., 2009
Connexin-43	F: ggctgctcatccccaactg R: gactgctcattctgctgctgg	325	57	Wen *et al*., 2008*a*
Kct-2	F: ctggctttcctggtggccattgt R: agggacggcatggcctcgttg	158	60	This study
Sox2	F: tcaacgctgttcctgatgta R: ggcacggtgctctggtagtg	941	55	Wen *et al*., 2009
KHV-TK	F: gggttacctgtacgag R: cacccagtagattatgc	409	52	Bercovier *et al*., 2005

Bmp4, bone morphogenetic protein 4; Kct-2, keratinocyte-associated transmembrane protein 2; KHV, koi herpesvirus; TK, thymidine kinase.

in 10% bovine serum albumin in PBS for 1 h and probed with a rabbit polyclonal antibody against Pax-6, Cx43, occludin, GATA-4 or DARPP-32 (each diluted 1:500). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20 000) was added to bind the primary antibody, which was then visualized by using chemiluminescence (Immobilon Western, Millipore). The extract of snubnose pompano [*Trachinotus blochii* (Lacépède 1801)] brain (SPB) cells (Wen *et al*., 2010) was used as positive control.

RT-PCR ANALYSES

RT-PCR was performed as described previously (Wen *et al*., 2010). The total RNA in the KF-101 cells at passages 70–85 was isolated using a blood and culture cell total RNA isolation kit (Favorgen; www.favorgen.com) according to the manufacturer's protocol. The RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm. RNA (5 μg or 5 μl per sample) was used to generate cDNA using an Moloney murine leukemia virus reverse-transcriptase (M-MLV-RT) kit (Promega; www.promega.com).

PCR amplifications were performed using Taq DNA Pol Master Mix (Ampliqon; www.ampliqon.com). Table II lists the primer sets for Bmp4, Cx43, Kct-2 and Sox2. Primers specific for Kct-2 were designed according to the sequence of *Oreochromis niloticus* (XM_003451401) by using the Primer-basic local alignment search tool (BLAST) tool of NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast). Into each 0·2 ml thin-walled PCR tube, the following components were added: 1 μl complementary (c)DNA, 1 μM of each of the forward and reverse primers and 12·5 μl of Master Mix, according to the manufacturer's protocol. The PCR was conducted under the following conditions: 30 cycles of denaturation at 95° C for 1 min; annealing (Table II) for 30 s; extension at 72° C for 1 min and final extension at 72° C for 7 min. The PCR products were electrophoresed on a 1·5% agarose gel and then purified using the gel purification kit (Favorgen) according to the manufacturer's protocol. Sequencing of the DNA fragments was performed commercially (Genomic BioSci & Tech; www.genedragon.com.tw). Sequences were identified using BLASTn (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

VIRUS SUSCEPTIBILITY AND CYTOPATHIC EFFECT

The KF-101 cells were tested for their susceptibility to the following fish viruses: grouper nervous necrosis virus GNNV-K1, iridovirus GSIV-K1 (Wen *et al*., 2008*b*), eel herpesvirus HVA980811 (Chiayi, Taiwan isolate), koi herpesvirus Taiwan KHV-T and chum salmon [*Oncorhynchus keta* (Walbaum 1792)] virus (CSV) (Winton *et al*.,

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1981). The cells were seeded into a six-well tissue culture plate and incubated at 25° C overnight. The medium was discarded, and then 0·2 ml of the virus solution was inoculated onto the cells and allowed to adsorb for 1 h. The medium was then replaced with a fresh L-15 medium without FBS. The infected cell monolayers were incubated at 25° C and were examined daily for a possible cytopathic effect (CPE). GBC1 cells were used as a positive control for GNNV and HVA. GBC4 cells were used as a control for GSIV and CSV (Wen *et al*., 2008*b*) and KFC was used as a control for KHV. Blind passages were performed every 7 days if a CPE did not occur in the infected cells.

KHV REPLICATION KINETIC ASSAY

The efficiency of KHV replication in the KF-101 cells was examined. The cells were placed in 75 cm^2 flasks at a density of 4×10^6 cells per flask, and were then infected with KHV-1 at a multiplicity of infection of 0·1. The virus solution was adsorbed for 1 h at room temperature and then the inoculum was removed. The monolayer was rinsed twice with the L-15 medium, and 20 ml of fresh L-15 medium was added and then incubated at 25° C. Samples of 1 ml of the infected cell medium were harvested at 0, 1, 2, 3, 5, 7, 9, 11, 13 and 15 days after viral inoculation. The titrations were performed in 96-well tissue culture plates, and the experiment was repeated twice. KHV DNA was extracted from the samples by using a viral nucleic acid extraction kit (Favorgen), and was detected by using a PCR assay that utilized thymidine kinase primers (Table II) under the conditions described previously (Bercovier *et al*., 2005).

RESULTS

CELL MORPHOLOGY AND GROWTH CHARACTERISTICS

Two weeks after the start of primary culture, a confluent monolayer was obtained. The morphology of cells from the fin of *C. carpio* was principally fibroblast-like prior to confluence [Fig. 1(a)]; the cells, however, became uniformly epithelioid at the confluence and showed contact inhibition [Fig. 1(b)]. The cells were subcultured at a split ratio of 1:2 or 1:3 at intervals between 4 and 7 days. These cells were designated as the KF-101 continuous cell line.

The KF-101 cells at passages 60–70 were analysed for growth characteristics. The growth curve of KF-101 cells in 10% FBS L-15 growth medium at 25° C is shown in Fig. 1(c). Some cells were damaged after trypsin digestion, which resulted in a growth latency of >2 days. At the log phase, the cell population doubled in *c*. 41 h. The KF-101 cells were subcultured >90 times after initiation, and showed morphological and growth characteristics that were similar to those of the early passages.

IMMUNOCYTOCHEMICAL CHARACTERIZATION

Immunostaining showed that the KF-101 cells were epithelial cells expressing keratin, Cx43 and occludin, but not vimentin [Fig. 2(a), (b)]. An unexpected finding was that neither of the junction proteins was specifically localized at the junctions, but appeared throughout the cells. Overall, the results confirmed that the KF-101 cells are epithelial cells. Because the KF-101 is derived from fin tissue, the cells were hypothesized to be the progenitors of epidermal cells. To test this hypothesis, the KF-101 cells in this study were stained with the stem cell proteins Pax-6 and Sox2. The Pax-6 staining appeared in the nuclei and the cytoplasm [Fig. 2(c)]. By

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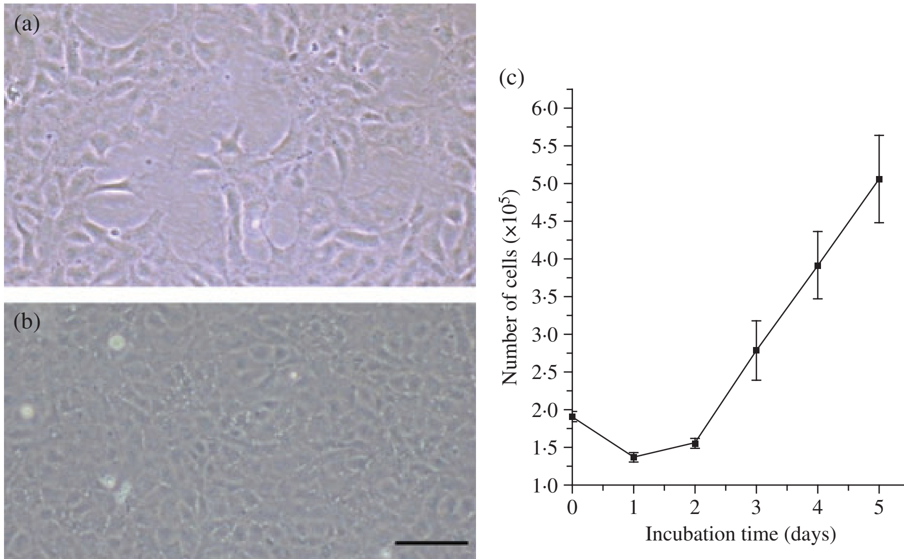

FIG. 1. (a) KF-101 cells at subconfluence, isolated fibroblast-like cells were observed. (b) Confluent monolayer of the KF-101 cells: the cells showed epithelial-like connections with neighbour cells and contact inhibition. Bar = 200 µm. (c) Growth curve of the KF-101 cells in 10% foetal bovine serum (FBS) L-15 medium at 25° C (means ± S.D., $n = 2$).

contrast, Sox2 staining was scarce [Fig. 2(d)]. Pax-6 and Sox2 are transcription factors involved in brain, eye and olfactory development; these proteins occur in progenitor cells (Hever *et al*., 2006; Guo *et al*., 2010; Georgala *et al*., 2011).

To test whether the KF-101 cells were neural progenitor cells, the cells were stained with various antibodies, including anti-A2B5, anti-brain lipid-binding protein, anti-DARPP-32, anti-galactocerebroside, anti-glial fibril acidic protein, anti-Olig2 and anti-tyrosine hydroxylase. Previous studies have used these antibodies to identify fish neural progenitor cells *in vitro* (Wen *et al*., 2008*a*, 2009, 2010). Apart from DARPP-32, all of the staining test results were negative. DARPP-32 is used to identify dopaminergic neurons, and various densities of DARPP-32 staining were observed at different cell cycle stages. DARPP-32 was observed in both the nuclei and the cytoplasm; staining was intense in the nucleus during the mitotic interphase and became heavy at the prophase [Fig. 3(a)]. Dense staining was observed throughout the cell at the prometaphase, but was heavier in the nucleus than in the cytoplasm [Fig. 3(b), (c)]. At the metaphase, the stains were observed in two parts dividing by chromosomes [Fig. 3(d)]. Overall, the quantity of DARPP-32 labelling was less at the anaphase and telophase [Fig. 3(e), (f)] when compared with those at the prophase and metaphase.

To investigate whether the KF-101 cells were derived from the mesendoderm, the cells were stained with an anti-GATA-4 antibody. Unexpectedly, the expression levels and locations of GATA-4 varied at different cell cycle stages. Isolated GATA-4 labels were observed around the centrosome or microtubule organization centre (MTOC) in the cells at the interphase [Fig. 4(a)]. By contrast, heavy stains were observed in the nuclei of the cells at the prophase and were distributed throughout the cytoplasm at the prometaphase [Fig. 4(b)–(d)] and metaphase [Fig. 4(e)–(g)].

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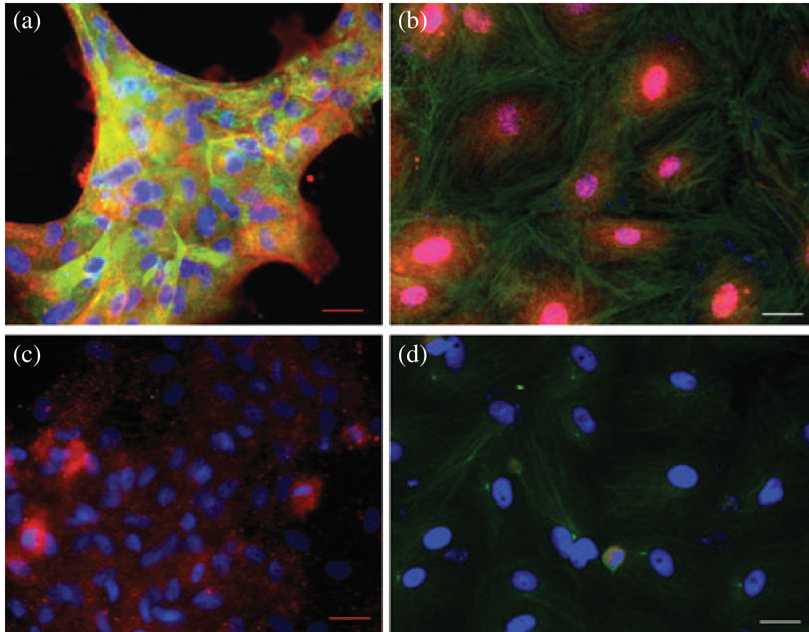

FIG. 2. KF-101 cells were double labelled with the following antibodies: (a) mouse monoclonal anti-keratin and polyclonal rabbit anti-connexin-43, (b) mouse monoclonal anti-vimentin (and polyclonal rabbit anti-occludin), (c) mouse anti-actin and rabbit anti-Pax-6 and (d) mouse anti-tubulin and rabbit anti-Sox2. Mouse antibodies were labelled with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (green), and the rabbit antibodies were labelled with Alexa Fluor 568-conjugated anti-rabbit IgG (red). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Bar = 20 µm.

The labels appeared at the midbody and the two polar bodies in the cells during the anaphase and telophase [Fig. 4(h)–(j)]. The quantity of GATA-4 decreased gradually in the cells after the metaphase.

IMMUNOBLOTTING DETECTION

Immunoblotting was used to confirm the identity of proteins that were detected by immunofluorescence microscopy. The immunoreactive bands in the SPB cell extracts with molecular masses of *c*. 47, 43, 65, 48 and 32 kDa were identified as Pax-6, Cx43, occludin, GATA-4 and DARPP-32, respectively [Fig. 5(a)]. Additional anti-GATA-4 and anti-DARPP-32 reactive bands were observed, however, but their identities were unclear. When compared with the SPB extract, the extract of KF-101 contained abundant Cx43 and showed three additional bands. The bands with a mass >43 kDa were surmised to be the trimer and dimer of Cx43, and the bands with a lower mass were the degraded protein. The KF-101 cells showed minimal GATA-4 when compared with SPB cells. Although SPB displayed a major band of GATA-4 with a mass of 48 kDa, in KF-101, the major anti-GATA-4 reactive protein had a mass of *c*. 37 kDa. The pattern of DARPP-32 immunoreactive proteins in KF-101 also differed from that of SPB. Additional studies are required to confirm the identity of the immunoreactive proteins in KF-101.

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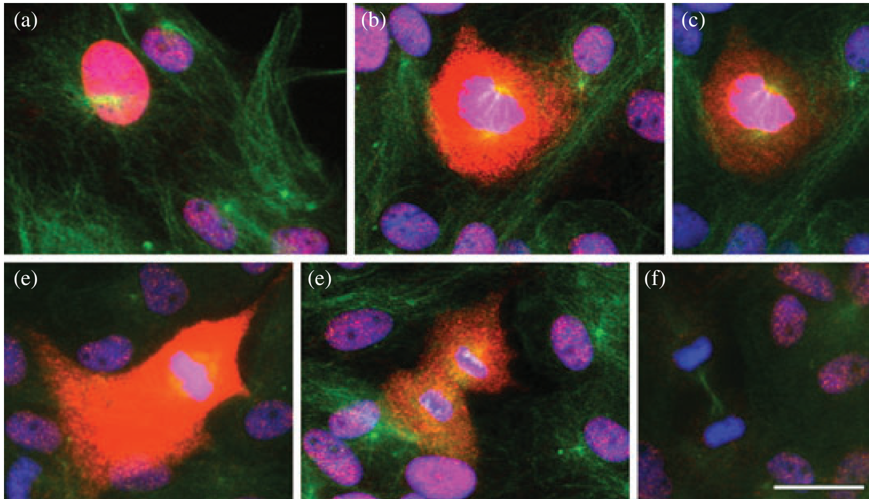

FIG. 3. KF-101 cells were double stained with mouse anti-tubulin (green) and rabbit anti-DARPP-32 (red) antibodies; the secondary antibodies and nuclei stains are shown in Fig. 2. Labelling of DARPP-32 was intense in the nuclei but varied across cell-cycle stages. (a) Nucleus at the mitotic prophase showed a higher density than at the interphase. (b) At the prometaphase, both the nucleus and the cytoplasm were highly stained. (c) Cell at the prometaphase, but with decreased exposure time, when compared with (b); staining in the nucleus was greater than in the cytoplasm. (d) Cell at the metaphase, with staining distributed throughout the cell. (e) Cell at the telophase, showing decreased DARPP-32 staining, when compared with that at the metaphase. (f) Cells at cytokinesis showed minimal staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Bar = $20\,\mu m$.

RT-PCR DETECTION

Agarose gel electrophoresis of the RT-PCR products revealed bands for Bmp4, Kct-2 and Cx43 [Fig. 5(b)]; the observed sizes corresponded to the predicted values (Table II). The BLAST programme revealed that the nucleotide sequences (including the primer) of Bmp4 and Cx43 achieved 98 and 96% congruence with the respective common carp Bmp4 (HQ446455) and Cx43 (AY008286). The results suggested that the KF-101 cells were derived from *C. carpio*. The nucleotide sequence of Kct-2 achieved 85 and 80% congruence with the respective salmon Kct-2 (NM_001139860) and nil tilapia (*Oreochromis* sp.) Kct-2 (XM_001521374). The nucleotide sequences of Bmp4 and Cx43 were deposited in GenBank (NCBI) with the following accession numbers: JX446582 for Bmp4 and JX446581 for Cx43. The RT-PCR also revealed that the KF-101 cells displayed extremely low levels of Sox2 mRNA [Fig. 5(b)].

CPE AND REPLICATION CURVE OF KHV

A typical CPE, namely cytoplasmic vacuolization and syncytium, was observed in the KF-101 cells within 4 days after KHV inoculation at 25° C [Fig. 6(a)]. The PCR analysis [Fig. 6(b)] and replication curve [Fig. 6(c)] showed that the viral yield increased over time for 7 days post-inoculation; thereafter, the infectivity of progeny virus decreased. No CPEs were observed in the KF-101 cells infected with HVA, GSIV, GNNV or CSV (data not shown).

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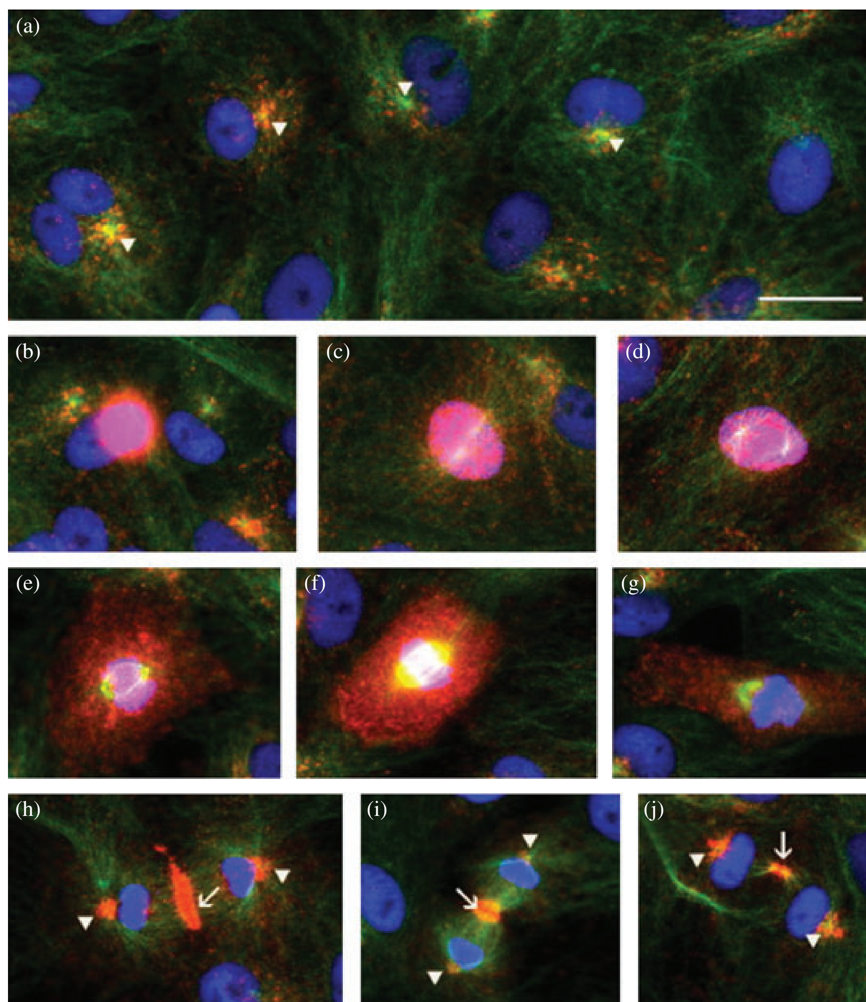

FIG. 4. KF-101 cells were double stained with mouse anti-tubulin (green) and rabbit anti-GATA-4 (red) antibodies. Staining of anti-GATA-4 was cell cycle-regulated and was associated with microtubules. (a) Labels are shown surrounding the centrosome (◁) at the interphase. (b–d) Labelling was dense in the nuclei during the prophase. (e–g) Cells during the prometaphase and metaphase, showing staining that diffused into the cytoplasm. At the late metaphase (g), the staining was less than at the early metaphase. (h) Labels are shown at middle plate (⇐) and at the two asters (◁) in the cell at the telophase. (i and j) Labels at the middle body (⇐) decreased following cytokinesis. Bar = 20 µm.

DISCUSSION

Studies on several cell lines obtained from *C. carpio* fin tissue have been published, including KF-1 (Hedrick *et al*., 2000), KFC (Ronen *et al*., 2003), KCF (Pikarsky *et al*., 2004), KCF-1 (Dong *et al*., 2011) and CCF (Lakra *et al*., 2010). Apart from CCF, which displays epithelioid morphology, all of these lines appear fibroblast-like. Identifying cells according to their morphology, however, is of limited value because epithelial cells can transform into fibroblast-like mesenchymal cells, and *vice versa*

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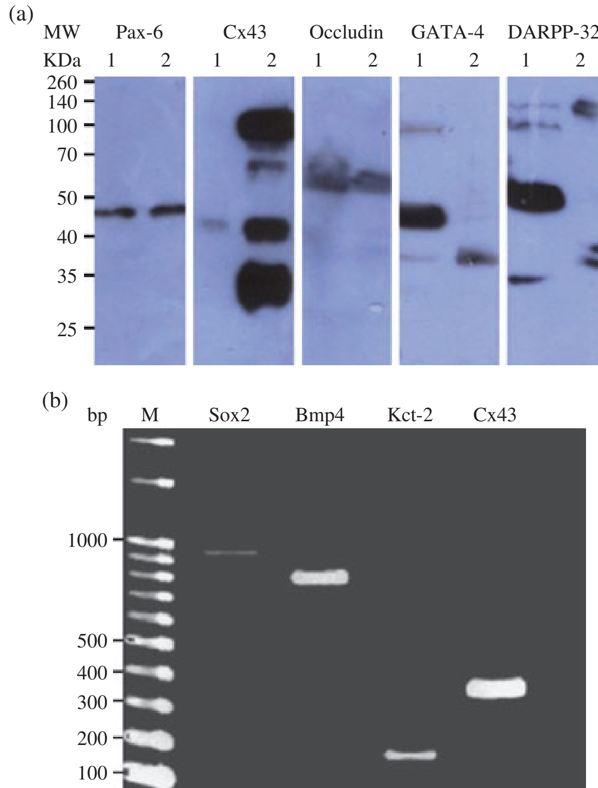

Fig. 5. (a) Reactivity of snubnose pompano (*Trachinotus blochii*) brain (SPB) cell (lane 1) and KF-101 cell (lane 2) extracts with the following antibodies: anti-Pax-6, anti-Cx43, anti-occludin, anti-GATA-4 and anti-DARPP-32 rabbit polyclonal antibodies. Labelling was visualized by chemiluminescence with horseradish peroxidase substrate. Predicted molecular masses (MW) for Pax-6, Cx43, occludin, GATA-4 and DARPP-32 were 47, 43, 65, 48 and 32 kDa, respectively. Each lane was loaded with 10 µg of protein. (b) RT-PCR analyses of KF-101 cells to detect transcripts for Sox2, Bmp4, Kct-2 and Cx43. Amplified products were cloned and sequenced to verify the specificity of the PCR reaction.

(epithelial–mesenchymal transition) during cell development. Fish fin tissue has been shown to contain the following nine distinct lineage classes: epidermis, melanocyte or xanthophore, iridophore, intraray glia, lateral line, osteoblast, dermal fibroblast, vascular endothelium and resident blood (Tu & Johnson, 2011). Fin tissue provides a widely used source of cell lines.

KF-101 CELLS ARE EPIDERMAL PROGENITOR CELLS

In this study, a new cell line from the caudal fin of *C. carpio* was established and characterized. These KF-101 cells were fibroblast-like at subconfluence, but displayed an epithelial morphology at confluence. The KF-101 cells were epithelial cells because they expressed keratin, Cx43 and occludin, but not vimentin (which is the mesenchymal intermediate filament protein). The nuclei of the KF-101cells, however, displayed dense Pax-6, which is not observed in epidermal cells but occurs abundantly in the stem or progenitor cells of the eye, brain and olfactory system.

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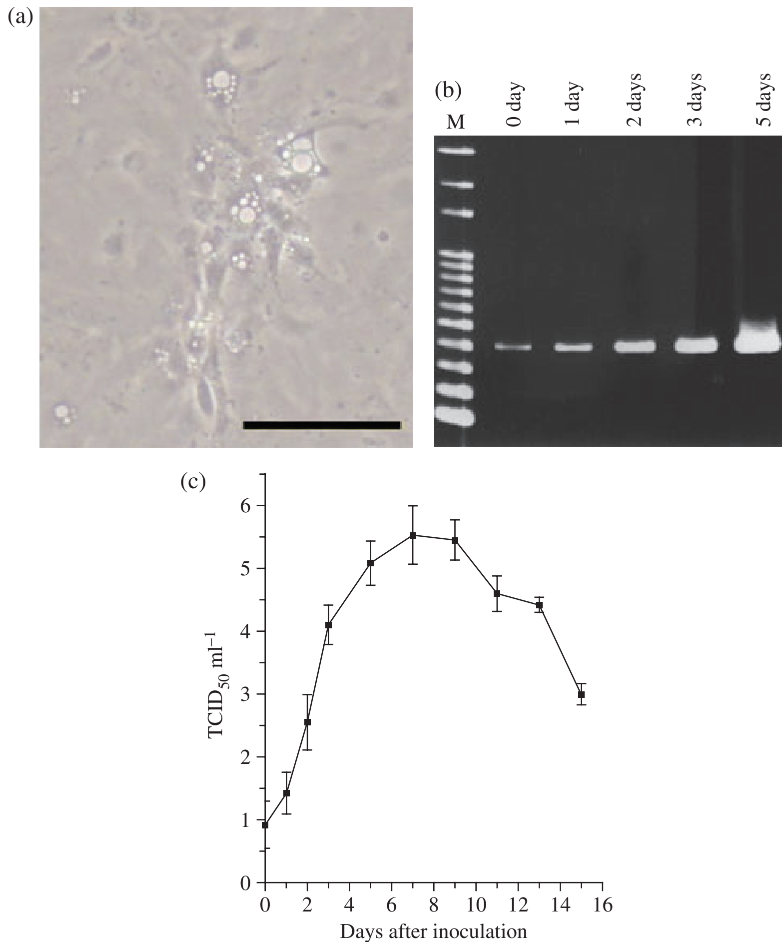

FIG. 6. (a) KF-101 cells showing cytoplasmic vacuolation 4 days post-infection with KHV at 25° C. Bar = 200 μm. (b) PCR analyses of KHV DNA expression over time. PCR product M: 100 bp ladder. (c) KHV replication curve in KF-101 cells incubated at 25° C. Viral titres were monitored over time. $TCID_{50}$, 50% tissue culture infective dose.

Pax-6 also occurs in mature neural cells such as astrocytes, oligodendrocytes and neurons (Plaza *et al*., 1995; Davis & Reed, 1996).

To confirm whether the KF-101 cells were neural epithelial cells derived from intraray glia or a lateral line, the cells were stained with a panel of antibodies previously used in the laboratory to characterize neural progenitor cells (Wen *et al*., 2008*a*, 2009, 2010). The results showed that the KF-101 cells are not neural cells. The question arises why KF-101 cells display Pax-6 if they are of epidermal origin. The KF-101 cells are hypothesized epidermal stem or progenitor cells, and that, *in vitro*, these cells acquire the ability to express Pax-6. This hypothesis is supported by the facts that keratinocyte stem cells *in vitro* can re-express Pax-6 and transdifferentiate into corneal epithelia (Blazejewska *et al*., 2009; Yang *et al*., 2009). In this study, the RT-PCR and immunostaining analyses showed that the KF-101 cells

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are epidermal progenitor cells originating from *C. carpio*. The ability of the cells to generate mature keratinocytes requires further study.

ANTI-DARPP-32 ANTIBODY CROSS-REACTED WITH THE PHOSPHATASE-1 (PP1) INHIBITOR-1 IN KF-101

In this study, the anti-DARPP-32 antibody (Ab51120; Abcam; www.abcam.com) densely labelled in the nuclei of the KF-101 cells was unexpected. DARPP-32 typically appears enriched only in the cytoplasm of dopaminoceptive neurons (Walaas *et al*., 1983). The antibody was considered cross-reactive with PP1 inhibitor-1 in the KF-101 cells, because the antibody was produced using the immunogen with an amino acid sequence close to RPTPA and similar to inhibitor-1 (Williams *et al*., 1986). In contrast with the limited distribution of DARPP-32 in the brain, inhibitor-1 is widely distributed in the brain and in various peripheral tissues (Hemmings *et al*., 1992).

This study determined that the labelling of anti-DARPP-32 accumulated in the nuclei of KF-101 cells during mitosis. This accumulation might be linked to the activity of PP1. Inactive PP1 is required for G1/S transition, centrosome separation and chromosome condensation at the mitotic prophase, and nuclear envelope disruption at the prometaphase (Ceulemans & Bollen, 2004); thus, inhibitor-1 increases in these stages. The transition between the metaphase and the anaphase and the reassembly of the nuclear envelope require active PP1 (Ceulemans & Bollen, 2004; Bollen *et al*., 2009); thus, the level of inhibitor-1 decreases during mitotic exit. The labelling of anti-DARPP-32 fit the action of inhibitor-1.

ANTI-GATA-4 ANTIBODY CROSS-REACTED WITH THE GATA-3 IN KF-101

GATA transcription factors are zinc-finger DNA-binding proteins that control the development of diverse tissues by activating or repressing transcription. GATA-4 is expressed predominantly in endoderm- and mesoderm-derived tissues such as the heart, liver, lung, gonad and gut. GATA-4 is known to play a role in normal heart development in mammals (Molkentin, 2000). Until this study, GATA-4 has not been identified in ectodermal cells. By contrast, expressions of GATA-2 and GATA-3 have been reported in ectodermal neural and non-neural cells (Neave *et al*., 1995; Read *et al*., 1998; Sheng & Stern, 1999). Furthermore, GATA-3 is known to be involved in follicular and epidermal morphogenesis (Kaufman *et al*., 2003; Chikh *et al*., 2007; Sellheyer & Krahl, 2010). Results in this study suggest that the anti-GATA-4 antibody (Ab61170; Abcam) labelled the GATA-3 in the KF-101 cells, because of the strong similarity in amino acid sequences between these two proteins around the site (RLSAS) of the immunogen.

Previous studies have shown that GATA-3 is essential for the development of mammary epithelia (Kouros-Mehr *et al*., 2006). GATA-3 is also known to inhibit the expression of vimentin and the epithelial–mesenchymal transition, and to promote mesenchymal cell differentiation into epithelial cells (Yan *et al*., 2010). Lowered levels of GATA-3 promote breast cancer development (Kouros-Mehr *et al*., 2008); thus, GATA-3 has been suggested as a tumour suppressor in the breast. GATA-3 has been shown to occur in partial conjunction with both p63 isoforms, TAp63 and

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ΔNp63 (Asselin-Labat *et al*., 2007). The p63 group is one of the p53 family proteins that are transcription factors involved in both cancer and normal development. The ΔNp63 isoform is the most commonly expressed p63 type and accumulates in mitotic cells and decreases during mitotic exit (Hau *et al*., 2011). GATA-3 is suggested a cell cycle regulation factor in KF-101 cells.

In this study, the location and density of GATA-3 were regulated by the cell cycle. GATA-3 appeared in sparse spots surrounding the centrosome at the interphase, accumulated abundantly in the nucleus at the prophase and prometaphase, was diffused throughout the cytoplasm at the metaphase and gathered at the midbody and two asters during the anaphase and cytokinesis. Many proteins are known to be involved in the formation of the midbody; these include acetylated α-tubulin, γ-tubulin, Aurora-A, Aurora-B, breast cancer susceptibility protein BRCA2, centrosome protein Cep55, collapsin response mediator protein-1 (CRMP-1), mitogen-activated protein (MAP) kinases, mitotic kinesin-like protein 1 (MKLP-1) and septin 1 (Zecevic *et al*., 1998; Inaguma *et al*., 2001; Daniels *et al*., 2004; Fabbro *et al*., 2005; Chen *et al*., 2009; Mondal *et al*., 2012). By contrast, GATA factors have not been reported to be involved in midbody formation. The functions of GATA-3 in KF-101 cells require further study.

APPLICATIONS OF KF-101

The KF-101 cells were characterized epidermal progenitor cells obtained from *C. carpio* fin. The cells were susceptible to koi herpesvirus and produced a typical CPE, when compared with other cell lines from *C. carpio* fin (Hedrick *et al*., 2000; Pikarsky *et al*., 2004; Dong *et al*., 2011). The messenger (m)RNA and protein expressions of Bmp4, Cx43, occludin, GATA-3, PP1 inhibitor-1, Pax-6 and Sox2 were initially discovered in *C. carpio* cells *in vitro*. Numerous cell lines from the *C. carpio* fin (namely KF-1, KFC, KCF, KCF-1 and CCF) that are cultured using Eagle's minimal essential medium (EMEM) or Dulbecco's modified Eagle medium (DMEM) require CO_2 supplementation; by contrast, the KF-101 cells grew effectively in 10% FBS containing L-15 medium, without additional CO_2. The KF-101 cells doubled in number within *c*. 41 h under optimal conditions, and could be stored well in liquid nitrogen. The KF-101 line is well characterized, when compared with other *C. carpio* cell lines, and can be used in studies on the development, defence and tumourigenesis of the epidermis and in the field of aquatic toxicology. Because they are epidermal cells, KF-101 cells might also prove useful in the study of mechanisms for entering pathogenic bacteria and the attachment and growth of epidermal parasites.

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