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ORIGINAL PAPER

Development and characterization of a new marine fish cell line from turbot (*Scophthalmus maximus*)

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Abstract A new marine fish cell line, TK, derived from turbot (*Scophthalmus maximus*) kidney, was established by the method of trypsin digestion and subcultured for more than 50 passages over a period of 300 days. The TK cells were maintained in Minimum Essential Medium Eagle (MEM) supplemented with HEPES, antibiotics, fetal bovine serum (FBS), 2-Mercaptoethanol (2-Me), and basic fibroblast growth factor (bFGF). The suitable growth temperature for TK cells was 24°C, and microscopically, TK cells were composed of fibroblast-like cells. Chromosome analysis revealed that the TK cell line has a normal diploid karyotype with $2n = 44$. Two fish viruses LCDV-C (lymphocystis disease virus from China) and TRBIV (turbot reddish body iridovirus) were used to determine the virus susceptibility of TK cell line. The TK cell line was found to be susceptible to TRBIV, and the infection was confirmed by cytopathic effect (CPE) and transmission electron microscopy, which detected the viral particles in the cytoplasm of virus-infected cells. Finally, significant green fluorescent signals were observed when the TK cells were transfected with pEGFP-N3 vector, indicating its potential utility for fish virus study and genetic manipulation.

Keywords Turbot · *Scophthalmus maximus* · Kidney cell line · TK · Karyotype · Virus susceptibility

Introduction

The development of animal cell lines provide an important tool to study virology, immunology, genetics, oncology, developmental biology, toxicology, clinical medicine and biotechnology (Hightower and Renfro 1988; Bonewald 1999; Ledermann 2000; Petersen et al. 2003; Donato et al. 2008; Dash et al. 2008). Since the 1960s, more than one hundred fish cell lines have been established and used for the study of virology, immunology and genetics (Fernández et al. 1993; Fernández-Puentes et al. 1993; Fryer and Lannon 1994; Chang et al. 2001; Chen et al. 2003a, b, 2005; Hong et al. 2004; Zhou et al. 2007; Parameswaran et al. 2007).

Turbot (*Scophthalmus maximus*) is a widely cultivated marine fish species with high economic value in Europe and China. However, intensive aquaculture of this fish species has resulted in outbreaks of diseases caused by several virus agents such as turbot iridovirus (Bloch and Larsen 1993), infectious pancreatic

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necrosis virus (IPNV) (Novoa et al. 1993), turbot herpesvirus (Hellberg et al. 2002), turbot nodavirus (Johansen et al. 2004) and viral hemorrhagic septicemia virus (VHSV) (Nishizawa et al. 2006) with high mortalities. In China, reddish body syndrome (RBS) is one viral disease causing mass mortality of turbot (*S. maximus*). An iridovirus was isolated from diseased turbot with RBS and designated TRBIV (turbot reddish body iridovirus) (Shi et al. 2004). Little is known about the epidemiology or characteristics of the virus in turbot due to lack of suitable cell lines. Thus, a susceptible cell line is urgently required in turbot for isolation and identification of viruses that cause viral diseases in this species.

Recently, several cDNA libraries of turbot immune-relevant tissues have been constructed and more than one thousand genes were identified (Meng et al. 2008; Pardo et al. 2008; Park et al. 2009), while little is known about the detailed functions formation of these genes. The establishment of suitable cell lines could help to analyze the function of these important immune genes.

So far, several cell lines have been developed from turbot (Tocher et al. 1989; Fernández-Puentes et al. 1993; Chen et al. 2005). However, there are no detailed reports of the application of virus susceptibility and foreign DNA transfection in these cell lines. And importantly, still no reports existed about the establishment of turbot immune tissues such as kidney, spleen and so on. In this paper, we described the establishment and characterization of the turbot (*S. maximus*) kidney cell line (TK) for the first time. Further, the susceptibilities of the TK cells to LCDV-C (lymphocystis disease virus from China) and TRBIV were assessed. The expression of a foreign gene in this new cell line was also examined. The results showed that the TK is susceptible to TRBIV and suitable for foreign gene transfection, suggesting its potential role in virus and gene function study.

Materials and methods

Primary cell culture and subculture

A healthy turbot weighing 150 g was obtained from Haiyang Fisheries Company in Yantai, Shandong Province of China. The fish was anesthetized with MS-222 and then wiped with 70% alcohol. The kidney was removed and placed in Minimum Essential Medium Eagle (MEM) containing antibiotics (1000U ml^{-1} of penicillin, 1000U ml^{-1} of streptomycin). The kidney tissue was minced thoroughly with scissors and then digested with 0.5% trypsin–EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA-4Na per milliliter of 0.9% sodium chloride, sterile-filtered) for 30 min. The residual liquids filtered with 200-mesh nylon gauze were transferred into 25-cm^2 cell culture flasks containing 3 ml of MEM with 20 mM HEPES, 20% fetal bovine serum (FBS), 0.05% 2-Mercaptoethanol (2-Me), and 2 ng ml^{-1} recombinant human basic fibroblast growth factor (bFGF). The primary cells were maintained at 24°C. Half of the growth medium was changed every 4 days for the first 3 weeks.

When a complete monolayer had formed in primary culture, confluent cells were digested with 0.25% trypsin–EDTA solution and transferred into a fresh 25-cm^2 flask. Initially, culture cells were maintained in MEM with 20% FBS; however, after 8 passages the concentration of FBS in MEM was reduced to 10%.

The growth curve of TK cells

To analyze the growth characteristics of the TK cells, 2×10^4 cells at passage 15 were seeded in every well of five wells in a 12-well plate with MEM containing 10% FBS and three repeated plates were incubated at 15, 20, 24, and 30°C for growth curve tests. At 1, 2, 3, 4, and 5 days after inoculation, cells in three wells for each different temperature were trypsinized, and cell numbers were measured microscopically using a hemocytometer.

Chromosome analysis

Chromosome preparation for TK cells was carried out as described with some modifications (Earley 1975). Briefly, the TK cells at passage 12 were inoculated into 25-cm^2 culture flasks and incubated at 24°C for 24–36 h. Colchicine was added into the cells with a final concentration of 0.5 μg per ml. After 3 h incubation, the cells were harvested, treated with 5 ml of 0.075 M KCl for 25 min, and then pre-fixed for 2 min in 1 ml of Carnoy's fixative (methanol: acetic acid = 3:1). After 5 min centrifugation at

1,500 rpm, the cell pellets were fixed with cold Carnoy's fixative for 15 min. After centrifugation, cells were resuspended in 0.5 ml Carnoy's fixative at −20°C for overnight. The next day, the suspension was dropped on cold glass slides, air dried, and stained with 5% Giemsa (pH 6.8) for 25 min. Finally, the slides were observed and photographed under Nikon Eclipse 80I fluorescence microscope. One hundred cells at metaphase were counted, and chromosome karyotype was analyzed according to the method of Levan (1964).

Virus challenge assay

The viruses LCDV-C and TRBIV, isolated from diseased Japanese flounder and turbot, respectively, were used to determine the TK cells virus susceptibilities. The preparation and confirmation of the two viruses was performed as described in Shi et al. (2004, 2008) and the titration value of LCDV-C and TRBIV determined based on TCID50 assay was 10^2 $TCID_{50}$ ml^{-1} and 10^3 $TCID_{50}$ ml^{-1}. One day before virus infection, cells at passage 17 were inoculated into 25-cm^2 flasks. The infection was carried out by adding 1 ml LCDV-C and TRBIV virus suspension into the cells, and 1 h later, the virus solution was replaced with new medium. After infection, the cytopathic effect (CPE) was observed using a Nikon ECLIPSE TE2000-U fluorescence microscope every day. Cells exhibiting CPE and the virus uninoculated cells were collected for electron microscopy observation.

Electron microscopy

The virus treated and uninoculated cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 4 h at 4°C, rinsed in PBS buffer (0.1 M, pH 7.4) for 10 min three times, and then postfixed with 1% osmium tetroxide in cacodylate buffer (0.1 M, pH 7.4) for 2 h. After being rinsed three times in PBS buffer (0.1 M, pH 7.4) for 10 min, the specimens were dehydrated in graded ethyl alcohol (30, 50, 70, 90, 100%) and embedded in Epon812 epoxy resin. Ultra-thin sections were cut on a Reichert-Jung Ultracut-E microtome with a diamond knife, mounted on copper grids, stained with 2% uranyl acetate-lead citrate, then examined and photographed under a JEOL JEM-1200EX transmission electron microscope.

Cell transfection with GFP reporter gene

A day before DNA transfection, the TK cells at passage 12 were plated at a density of 3×10^5 cells per well in a 6-well plate at 24°C. More than eighty percent confluent monolayers were transfected with pEGFP-N3 vector (Clontech) using lipofectamine 2000 (Invitrogen). Briefly stated, 5 μl lipofectamine 2000 was added into a 1.5-ml centrifuge tube containing 245 μl MEM without FBS. In another centrifuge tube 10 μl pEGFP-N3 (100 ng μl^{-1}) was added with 240 μl MEM without FBS. After 5 min, the aforementioned two solutions were mixed together and kept for 25 min at room temperature. Then 500 μl mixtures were added into a well of the 6-well plate containing 2 ml MEM without FBS and cultured at 24°C for 6 h. Then the medium was replaced with normal medium. After 36 h, the green fluorescence signals were observed under a Nikon ECLIPSE TE2000-U fluorescence microscope.

Results

Primary culture and subculture

By the trypsinized method, primary monolayer cells were obtained from the kidney of turbot (*S. maximus*) about 20 days after incubation. The TK cells are composed of fibroblastic cells (Fig. 1). During the initial 8 subcultures, a MEM medium containing 20% FBS was used and the cells were split at a ratio of 1:2 every 8 days. From passage 8 on, the cells were subcultured in the ratio of 1:2 every 4 or 5 days with MEM containing 10% FBS. To date, the TK cell line has been subcultured through more than 50 passages over about 300 days.

The biphasic growth curve of TK cell line in different temperatures

The TK cells were able to grow at temperature between 20°C and 30°C (Fig. 2) with passage 15 cells. The highest growth rate was obtained at 24°C, when the cell number reached 5.2×10^4 cells ml^{-1} after 5 days. No obvious cell growth was observed at 15°C in the cells.

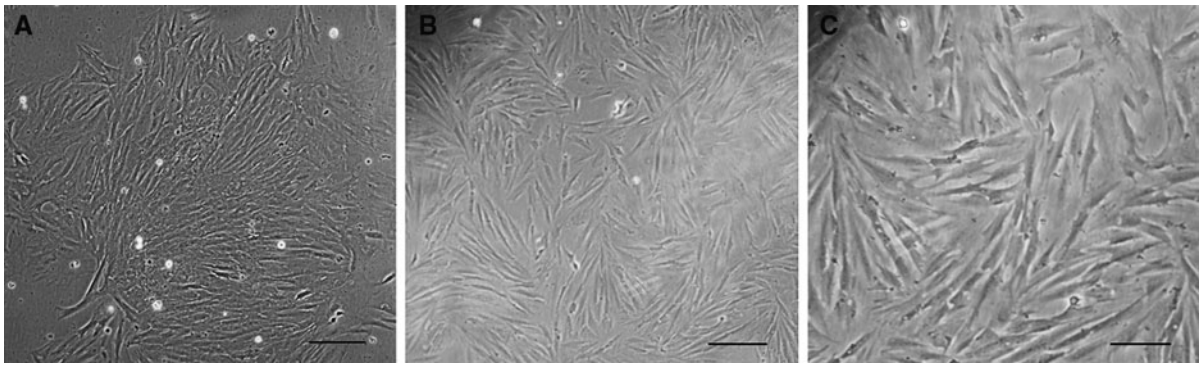

Fig. 1 Monolayer cells of the TK cell line. **a** The cells of primary passage. A region of cell colony has formed. **b**, **c** The cells at passage 40, which were composed of fibroblastic cells. *Scale bars* = **a** and **b** 100 μm, **c** 50 μm

The chromosome analysis of TK cells

The chromosome assay showed that the number of chromosome ranged from 26 to 84, nevertheless, the main chromosome number was 44, which occupy 68% in the 100 metaphase cells counted at passage 12 (Fig. 3a). The distribution was symmetrical, and the number of 2*n* values less than 44 was almost equal to that of 2*n* values greater than 44. The metaphase (Fig. 3b) with a normal diploid number displayed the normal karyotype morphology (Fig. 3c) consisting of 2 pairs of mediocentrics, 6 pairs of subtelocentrics, and 14 pairs of telocentrics ($2n = 4m + 12st + 28t$).

The virus susceptibility of the TK cells

The viruses TRBIV and LCDV-C were used to infect TK cells at passage 17. The cells inoculated with TRBIV (Fig. 4b) exhibited morphological changes including cell shrinkage and cell elongation, compared to the uninoculated control cells (Fig. 4a) after 5 days. There were no significant morphological changes observed in cells infected with LCDV-C.

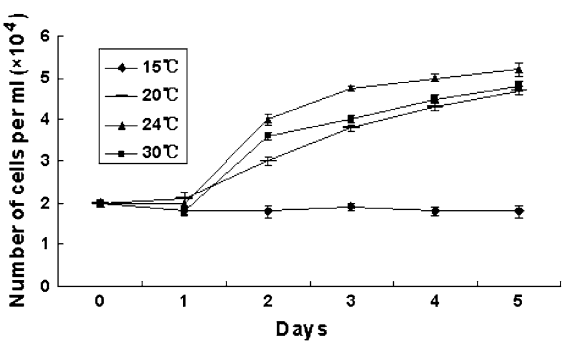

Fig. 2 The growth of TK cells at passage 15 at different temperatures between 20 and 30°C. The highest growth rate was obtained at 24°C, when the cell number reached 5.2×10^4 cells ml^{-1} after 5 days. No obvious cell growth was observed at 15°C in the cells

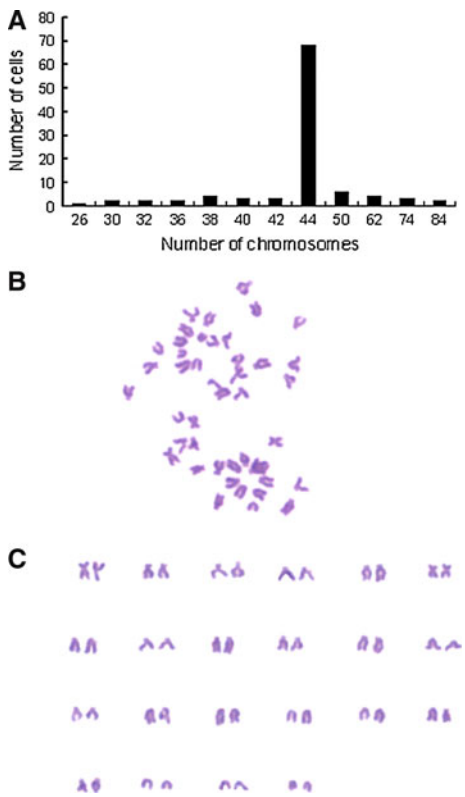

Fig. 3 Chromosome number distribution (**a**), metaphase (**b**) and diploid karyotype (**c**) of TK cells at passage 12. The main chromosome number was 44, which consisted of 2 pairs of mediocentrics, 6 pairs of subtelocentrics, and 14 pairs of telocentrics ($2n = 4m + 12st + 28t$)

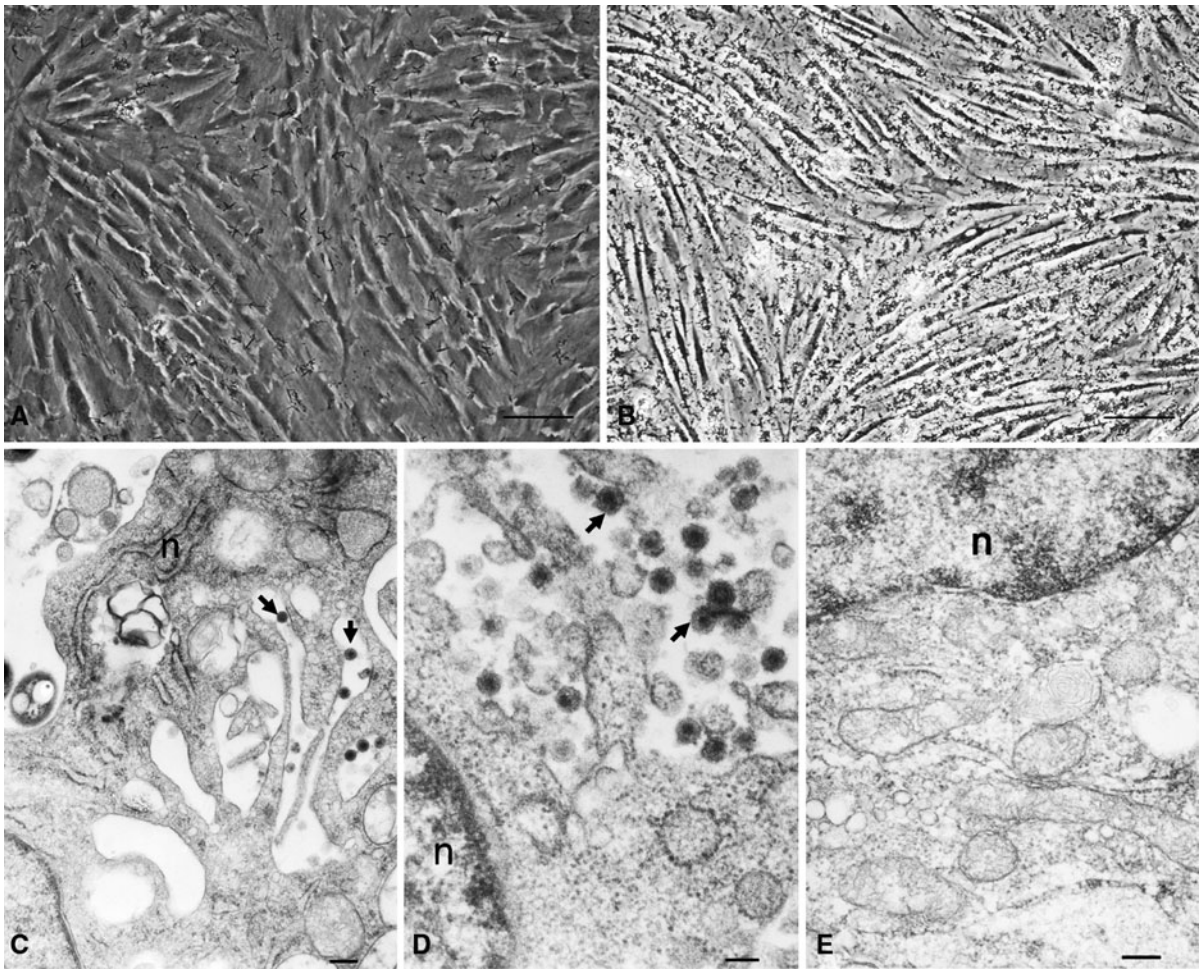

Fig. 4 The virus TRBIV infection in TK cells at passage 17. The cells inoculated with TRBIV (**b**) exhibited morphological changes including cell shrinkage and cell elongation, compared with the uninoculated control cells (**a**) after 5 days. **c**, **d** Revealed that virus particles (*arrows*) were scattered throughout the cytoplasm of cells infected with TRBIV. **e** The virus uninoculated TK cells. *Scale bars* = **a** and **b** 50 μm, **c** and **e** 200 nm, **d** 100 nm. *n* cell nucleus

Electron microscopy observation revealed that the virus particles were scattered throughout the cytoplasm of cells infected with TRBIV (Fig. 4c, d). Identical with the morphological observation, no virus particle was found in the cells infected with LCDV-C. The virus uninoculated cells were used as the negative control shown in Fig. 4e.

DNA transfection in TK cells

To determine the ability of the TK cell line to be transfected, the TK cells at passage 12 were transfected with 1 ug plasmid pEGFP-N3. Clear, multiple, and strong green fluorescence signals were observed at 36 h after transfection (Fig. 5). This indicated the reporter gene GFP could express in TK cells.

Discussion

In this study, a turbot (*S. maximus*) kidney cell line designated as TK was developed and characterized. The TK cell line has been subcultured for more than 50 passages over about 300 days.

The highest growth rate of the TK cell line was obtained at 24°C in MEM medium supplied with 10% FBS. The optimal temperature of TK cells was similar with turbot fin cell line (Fan et al. 2007), TEC (turbot

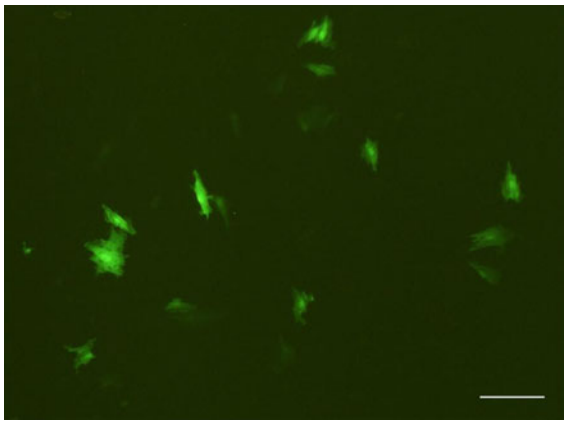

Fig. 5 The expression of GFP gene in TK cells at passage 12 transfected with pEGFP-N3. Scale bar = 50 μm

embryonic cell line) (Chen et al. 2005), and higher than 20°C of TV-1 cell line derived from posterior end of turbot (Fernández-Puentes et al. 1993). The different tissue origin may be the reason of different suitable growth temperature of turbot cell lines. As we know, the optimal temperature range for turbot aquaculture is 14–17°C, while several turbot cell lines including the TK cell line could obtain good growth condition at range of 20–25°C, which suggested the large temperature range of fish cell lines.

Karyotype analysis revealed that 68% of the cells possessed a diploid chromosome number of $2n = 44$, which was identical with the modal number of turbot (Bouza et al. 1994) and TEC (turbot embryonic cell line) (Chen et al. 2005).

Susceptibility of cell lines to virus infection is the basis for isolating and analyzing fish viruses. Although several cell lines such as TEC and turbot fin cell line was developed from the turbot, no virus susceptibility assay was carried out (Chen et al. 2005; Fan et al. 2007). Application of TK cell line for virus detection was assessed with LCDV-C and TRBIV viruses. The results showed that TK cell line exhibited no susceptibility to LCDV-C virus and high susceptibility to TRBIV. Electron microscopy detected a large number of virus particles in the cytoplasm of TRBIV-infected TK cells. This indicated that the TK cell line had different susceptibility to virus from different species. As we know, LCDV-C was isolated from Japanese flounder (Xu et al. 2000; Sun et al. 2000; Zhang et al. 2003), while TRBIV was isolated from turbot (Shi et al. 2004). In another parallel experiment, a southern flounder kidney cell line showed high sensitivity to LCDV-C (Wang et al. unpublished data). This experiment results were also identical with previous study (Iwamotoa et al. 2002) which found that of the 39 fish cell lines tested, only the HINAE cell line (Japanese flounder embryonic cell line) exhibited a cytopathic effect to JF-LCDV (Japanese flounder lymphocystis disease virus). These experiments both proved that fish LCDV had highly cell-specific susceptibility to different fish cell lines.

In the transfection experiment with pEGFP-N3 plasmid DNA, the TK cell line expressed the reporter gene efficiently and produced signals at 36 h after transfection. And the transfection efficiencies in TK cells was higher than in GSC (grouper snout cell line) (Zhou et al. 2007), SIMH (milkfish heart cell ine) and SIGE (grouper eye cell line) (Parameswaran et al. 2007), which suggested that the TK cells was more suitable for in vitro study of exogenous genes.

In conclusion, a new marine fish cell line TK was established from the kidney of turbot, which is the first report of a cell line derived from turbot immune-related tissues. In the preparation of this paper, this cell line has also been used into the sub-cellular location analysis and RNAi assay of turbot *hepcidin* and *grim19* (*gene associated with retinoid-interferon-induced mortality-19*) genes, as well as the proliferative activity of turbot IGF-1 (insulin-like growth factor 1) recombinant protein (Zhao et al. 2010), which further confirmed the utility of TK cell line in genetic study. And importantly, more fish virus' susceptibility to TK cell line need to be detected, which could progress the study of this cell line in virus isolation and cell–pathogen interaction study. Anyway, the present study suggested that the TK cell line could serve as a useful tool for fish virology and genetics.

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