A feeder-cell independent subpopulation of the PICM-19 pig liver stem cell line capable of long-term growth and extensive expansion

Neil C. Talbot · Thomas J. Caperna

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Abstract A method for the feeder-independent culture of PICM-19 pig liver stem cell line was recently devised, but the cell line's growth was finite and the cells essentially ceased dividing after approximately 20 passages over a 1 year culture period. Here we report the isolation, continuous culture, and initial characterization of a spontaneously arising feeder-independent PICM-19 subpopulation, PICM-19FF, that maintained replication rate and hepatocyte functions over an extended culture period. PICM-19FF cells grew to 90–98 % confluency after each passage at 2 week intervals, and the cells maintained a high cell density after 2 years and 48 passages in culture (average of 2.6×10^6 cells/T25 flask or 1×10^5 cells/cm²). Morphologically, the PICM-FF cells closely resembled the finite feeder-independent PICM-19 cultures previously reported, and, as before, no spontaneous formation of 3D multicellular ductules occurred in the cells' monolayer. Their bipotent stem cell nature was therefore not evident. Over extensive passage, cytochrome P450

(EROD) activity was maintained, although urea production was reduced on a per mg protein basis at later passages. Two other attributes of fetal hepatocytes, γ -glutamyl transpeptidase activity and serum-protein secretion, were also shown to be maintained by the PICM-19FF cells. The PICM-19FF cells therefore appear to have indefinite growth potential as a feeder-independent cell line and this should enhance the experimental usefulness of the cell line, in general, and may also improve its application to toxicological/pharmacological assays and artificial liver devices.

Keywords Cell · Culture · Feeder-cells · Hepatocyte · Liver · EROD · Urea

Introduction

The PICM-19 cell line was isolated from the spontaneous differentiation of primary cultures of pig embryonic stem cells, i.e., from the primary culture of the epiblast cells of the 8-day post-fertilization pig embryo or blastocyst (Talbot et al. 1994a). The parental PICM-19 cell line was demonstrated to differentiate into the two parenchymal cell types of the developing liver, fetal hepatocytes and bile duct epithelium, from monolayers of cells grown in vitro on STO feeder cells (Talbot et al. 1994a, b, 1996, 2002; Talbot and Caperna 1998). The PICM-19 cell line, and its derivative cell line, PICM-19H, exhibited serum protein production, inducible P450 γ activity and

N. C. Talbot (✉)
US Department of Agriculture, Beltsville Agricultural
Research Center, Animal Biosciences and Biotechnology
Laboratory, Bldg. 200, Rm. 13, BARC-East, Beltsville,
MD 20705, USA
e-mail: neil.talbot@ars.usda.gov

T. J. Caperna
US Department of Agriculture, Beltsville Agricultural
Research Center, Animal Biosciences and Biotechnology
Laboratory, Bldg. 200, Rm. 202, BARC-East, Beltsville,
MD 20705, USA

content, γ -glutamyltranspeptidase (GGT) activity, ammonia clearance, and urea production (Talbot et al. 1996, 2010a; Willard et al. 2010). The self-organizing, 3-dimensional, multicellular bile ductules formed by parental PICM-19 cells resembled bile ductules produced in vitro from the culture of fetal or adult pig liver tissue (Talbot et al. 1994b; Talbot and Caperna 1998). Also, the PICM-19 ductules expressed GGT at their apical cell surfaces (Talbot et al. 1996) and exhibited transcellular fluid transport to ductal lumens with in vivo-like kinetics in response to physiological levels of secretin (Talbot et al. 2002).

Recently, the PICM-19 cell line was demonstrated to grow and maintain hepatocyte function without contact with feeder-cells (Talbot et al. 2010b). The system consisted of growing the cells on a polymerized collagen I substrate in combination with an extracellular matrix overlay (Matrigel) using medium that was conditioned by STO feeder-cells. However, the feeder-independent cultures were finite in growth potential and slowly stopped growing after approximately 15 passages without direct contact with the feeder-cells (Talbot et al. 2010b). The isolation of PICM-19 cells capable of sustained growth without feeder-cells therefore became a goal since feeder-independent growth and function of the PICM-19 cells would enable their better use in such applications as artificial liver devices, toxicological/pharmacological assays, and the genetic engineering of the cells for agricultural and biomedical research initiatives.

Materials and methods

Cell culture

Medium was conditioned by STO feeder cells (CRL 1503, American Type Culture Collection, Rockville,

MD, USA) as previously described (Talbot et al. 2010b). PICM-19FF cells were propagated on polymerized collagen-coated T12.5 or T25 flasks (Falcon cultureware; BD Biosciences, Franklin Lakes, NJ and Greiner, Frickenhausen, Germany, respectively) as previously described (Talbot et al. 2010b). One day after each passage PICM-19FF cells were overlaid with a 1:25–1:50 dilution of Matrigel (BD Biosciences, Bedford, MA) as previously described for the finite feeder-independent cultures of PICM-19 cells (Talbot et al. 2010b). Also as previously described (Talbot et al. 2010b), growth of the cells was stimulated by daily refeedings of STO feeder-cell conditioned medium. Cell culture reagents were purchased from InVitrogen (Gaithersburg, MD, USA) and Hyclone (Logan, UT, USA).

For PICM-19 cell counts per flask (Table 1), PICM-19FF cells were passaged at a 1:3 split ratio, grown for ~ 2 week, and the number of cells per T25 flask is expressed as the average of 2 T25 flasks (except only 1 T25 flask was counted at P46). The total number of cells per T25 flask was determined by averaging the counts of 16 hemocytometer squares (1 mm^2) as previously described (Talbot et al. 2010a).

Cytochrome P450 EROD activity assay

The cytochrome P450 assay was performed as previously described (Talbot et al. 2010b; Willard et al. 2010). Briefly, early passage cells were duplicates from passage 23, 27, and 29 (12 flasks total, $n = 3$). Late passage cells were duplicates from passage 39 and 40 (8 flasks total, $n = 2$). T25 flask cultures of PICM-19FF cells were pre-incubated with $5 \mu\text{M}$ 3-methylcholanthrene (3MC) in STO-conditioned medium (CM) for 48 h to induce CYP1A1 activity.

Table 1 PICM-19FF cell counts over passage

Passage level	Post-passage (days)	Cells per T25 flask	Cells per cm^2
P25 off feeders	14	1.8×10^6	7.2×10^4
P44 off feeders	10	2.68×10^6	10.7×10^4
P45 off feeders	12	2.22×10^6	8.9×10^4
P46 off feeders	15	2.89×10^6	11.6×10^4

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Table 2 Influence of passage number on ethoxy resorufin-O-deethylase activity (A) and urea production (B) in PICM-19FF cells

(A) Resorufin (pmol/mg protein/30 min)

Passage	Direct activity (–βGase/AS)		Total activity (+βGase/AS)		Conjugated (%)	
	Control	3MC	Control	3MC	Control	3MC
Early	57 ± 16	4,751 ± 199	469 ± 63	7,792 ± 522	88.0 ± 1.8	38.9 ± 1.9
Late	26 ± 1	5,092 ± 1,314	321 ± 5	7,908 ± 1,519	91.8 ± 0.4	36.0 ± 4.3

(B) Urea N per 24 h

Passage	μg urea N/mg protein		Total μmole urea N/flask		NH ₄ Cl converted to urea (%)	Total protein (mg/flask)
	Control	2 mM NH ₄ Cl	Control	2 mM NH ₄ Cl		
Early	172 ± 25	244 ± 28	8.90 ± 1.6	13.3 ± 1	73 ± 13	0.736 ± 0.062
Late	90.4 ± 4.9*	116 ± 3.2*	10.9 ± 1.0	14.5 ± 1.7	60.4 ± 11.3	1.74 ± 0.11**

Data are mean ± SD, different by Students' *t*-test analysis, * *p* < 0.05 or ** *p* < 0.001

Cells were then exposed to Medium 199 with Hanks' salts without L-glutamine and containing 7-ethoxyresorufin (8 μM), dicumerol (10 μM), and bovine serum albumin for 30 min as described by Donato and coworkers (1993). The medium was harvested and the concentration of the fluorescent product, resorufin, was assayed in the absence ('Direct' fluorescence) or presence ('Total' fluorescence) of β-glucuronidase/arylsulfatase (Roche Applied Sciences, Mannheim, Germany) to determine the extent of EROD activity and conjugated EROD activity products, respectively. All reagents were from Sigma-Aldrich (St. Louis, MO, USA) and activity is presented as pmole product formed per 30 min/mg cell protein in cultures treated with and without 3MC (Table 2a).

Urea production assay

Urea production by the PICM-19FF cells after the addition of ammonia to the cell culture medium was performed as previously described (Talbot et al. 2010b). Briefly, early passage cells were duplicates from passage 24, 27 and 28 (12 flasks total, *n* = 3). Late passage cells were duplicates from passage 42 and 43 (8 flasks total, *n* = 2). Urea production was measured before and after the addition of 6 μmoles of NH₄Cl to the cell culture medium. Urea concentration in medium was determined colorimetrically based on a diacetyl monoxime reaction assay described by the World Health Organization (http://www.searo.who.int/en/section10/section17/section53/section481_1754.htm). Absorbance was determined at 540 nm and a standard curve was prepared (Urea nitrogen, Standard 535, Sigma-Aldrich).

Values from base medium were subtracted from experimental samples (Table 2b).

γ-Glutamyl transpeptidase (GGT) histochemical staining

Histochemical localization of GGT was determined by the method of Rutenberg et al. (1969). PICM-19FF cell monolayers were fixed for 2 min with ice-cold methanol just prior to the addition of substrate solution. For the stock solution of γ-Glutamyl-4-methoxy-2-naphthylamide (GMNA), 25 mg was dissolved in 0.5 ml dimethylsulfoxide (DMSO) with 0.5 ml of 1 N NaOH added. Thereafter, 9 ml of distilled water was added to give a final GMNA stock solution of 2.5 mg/ml, and it was stored at 4 °C for up to 3 days. The working substrate solution was made by mixing 1.0 ml of the GMNA stock solution with 5 ml of Tris buffer (0.1 M), pH 7.4, 14 ml of 0.85 % saline, 10 mg of Glycylglycine and 10 mg of Fast blue BBN (diazotized 4'-amino-2',5'-diethoxybenzylidene). The substrate solution was filtered through Whatman No. 1 filter paper just prior to use to remove any insoluble aggregates of substrate. PICM-19FF monolayers were stained for 5–10 min, washed in 0.85 % saline, and flooded with a 0.1 M solution of cupric sulfate for 2 min. The stained monolayers were given a final rinse in 0.85 % saline and photographed while under saline.

Two-dimensional electrophoretic analysis of conditioned medium (CM)

Two-dimensional (2D) electrophoretic analysis of PICM-19FF conditioned medium was performed as

previously described (Talbot et al. 2010a). Briefly, a T12.5 flask of confluent PICM-19FF cells was washed 4 times with serum-free medium [1:1 mix of Dulbecco's Modified Eagles Medium, high glucose formulation (DMEM) and Medium 199] and refed with 2 ml serum-free medium. After 72 h of culture, the CM was collected, subjected to low speed centrifugation to pellet cell debris, and the supernatant was frozen at -80°C . The serum-proteins in the CM sample were identified by comparison to previous 2D-gels of CM prepared from PICM-19H cells (Talbot et al. 2010a) and primary cultures of baby pig hepatocytes (Caperna et al. 2011) that were analyzed by mass spectroscopy (MALDI-TOF and LC-MS/MS).

Results

A population of feeder-independent PICM-19 cells, designated PICM-19FF, arose spontaneously from cultures of parental PICM-19 cells that had been grown without feeder-cells for approximately 15 passages. The emergence of the PICM-19FF cells was recognized by their enhanced growth and cell concentration per unit area in comparison with independent parallel cultures of parental PICM-19 cells being grown without feeder-cells. While all the other parallel culture flasks of PICM-19 under feeder-independent culture conditions irreversibly declined in growth rate until they could no longer be expanded in culture (Talbot et al. 2010b), the PICM-19FF cells continued to grow.

The capacity of the PICM-19FF cell line for long-term, feeder-independent growth was assessed. PICM-19FF cells were maintained and grown without feeder-cells on collagen I-coated tissue culture plastic with passages every 2 week at a 1:3 split ratio for over 2 years and a total of 50 passages off of STO feeder-cells. PICM-19FF growth potential was assessed by counting the total cells per flask after approximately 2 week of culture post-passage (Table 1) at an early passage level (P25) and at later passage levels (P44–P46). The PICM-19FF cells increased their growth potential over the 2 years of culture as demonstrated by their reaching average cell concentrations per flask of 2.6×10^6 cells/T25 flask, or 1×10^5 cells/cm², after nearly 50 passages at 1:3 split ratios compared to an initial, i.e., at P25, cell concentration of 1.8×10^6 cells/T25 flask or 7.2×10^4 cells/cm² (Table 1).

The morphology of the PICM-19FF cells was similar to the finite feeder-independent PICM-19 cells previously reported (Talbot et al. 2010b), i.e., cuboidal cells with prominent, centrally located nuclei that formed a monolayer, but without any multicellular ductal structures present (Fig. 1a). Also, similarly, the PICM-19FF cells had canalicular connections to each other that were responsiveness to glucagon. When glucagon was add to the medium (100 ng/ml) the cells' canaliculi enlarged within 10–15 min (Fig. 1b), presumably as a result of transcellular fluid transport into the canalicular space (Talbot et al. 1996). The absence of differentiation and remodeling into 3D bile ductules (cholangiocytes) would suggest that the PICM-19FF cells may no longer retain the bipotent stem cell potential of the parental PICM-19 cell line (Talbot et al. 1994a, 1996).

For the PICM-19FF cell line to be of use as an in vitro model of porcine liver it is critical that the cells retain the specialized metabolic functions of in vivo hepatocytes. Therefore, assessments of PICM-19FF hepatocyte detoxification functions, i.e., inducible P450 activity and urea production, were performed. PICM-19FF T25 cultures were assayed for inducible cytochrome P450 activity by inducing with 3MC and measuring EROD activity, i.e., conversion of 7-ethoxyresorufin (7-ER) to the highly fluorescent product resorufin. Total EROD activity was low (<500 pmol resorufin/mg cell protein/30 min) in the PICM-19FF cultures when not induced by exposure to 3MC (Table 2a). After induction with 3MC, PICM-19FF cells converted 7-ER to resorufin at rates on average of nearly 7.8×10^3 pmol/mg cell protein/per 30 min at early passages and at rates on average of 7.9×10^3 pmol/mg cell protein/per 30 min at late passages (Table 2a). The difference between early (P23, P27 and P29) and late passage (P39 and P40) PICM-19FF EROD activity was not significantly different ($p > 0.1$), indicating that the cell line's EROD activity was maintained over long periods in continuous culture. The relative amount of phase II activity in the PICM-19FF cultures was determined by evaluating the extent to which fluorescent activity was increased after treatment with β -glucuronidase/aryl-sulfatase, i.e., the difference between 'direct' and 'total' activity. This showed that under unstimulated conditions, where the total activity is low, approximately 90 % of the elaborated resorufin was in the conjugated form, and, when EROD activity was

Fig. 1 Phase-contrast micrographs ($\times 200$) of PICM-19FF cells at passage 22 off of STO feeder cells under plain medium in **a** and after 20 min in medium containing 0.1 $\mu\text{g/ml}$ glucagon in **b**. Note expanded biliary canaliculi between the PICM-19FF cells that were exposed to glucagon (arrows). Hoffman modulation micrograph ($\times 300$) of γ -glutamyl transpeptidase (GGT) histochemical staining of PICM-19FF at passage 36 off

of STO feeder cells (**c**); note intense GGT activity at apical membrane of biliary canaliculi (arrows; Talbot et al. 1996). Colloidal Coomassie Blue stained two-dimensional electrophoresis gel of 72 h conditioned medium from a confluent PICM-19FF culture at passage 36 off of STO feeder cells (**d**); note typical profile of secreted serum-proteins (Talbot et al. 2010a, b)—several are labeled including alpha-fetoprotein (AFP)

increased by 3MC induction, nearly 40 % of the resorufin end-product was conjugated. Phase II activities also demonstrated little change over passage (from P23 to P40; Table 2a).

Urea production by the PICM-19FF cells in the presence of added ammonia was assayed at early (P24, 27 and P28) and late passage (P42 and P43). PICM-19FF cells cultured in a glutamine free-medium containing glucagon maintained basal urea production similar to that previously observed with PICM-19 cells cultured off feeder-cells (Table 2b; Talbot et al. 2010b). When 2 mM NH_4Cl ($\sim 6 \mu\text{mol/flask}$) was added to the cultures PICM-19FF cells synthesized and secreted more urea (Table 2b). Late passage PICM-19FF cells produced significantly ($p < 0.05$) less urea (90 μg urea N/24 h) than early passage cells (172 μg urea N/24 h) on a per mg cell protein basis. Urea production was stimulated by 29 % following addition of 2 mM NH_4Cl for 24 h in early passage cells and by 22 % in the late passage cells (Table 2b).

As determined on a per flask basis, early PICM-19FF cells converted ~ 73 % of added ammonia N to urea N within 24 h, while late passage cells converted ~ 60 % of added ammonia N to urea N within 24 h. This difference was not significant ($p > 0.05$), and, on a per flask basis, early and late passage PICM-19FF cells converted a substantial amount of the added ammonia to urea.

Two other fetal hepatocyte functions were assayed in the PICM-19FF cell line; the expression of GGT in the cells' biliary canaliculi and the secretion of serum-proteins (Talbot et al. 1996, 2010a, b). Histochemical staining for GGT activity demonstrated that the PICM-19FF cells still expressed GGT (Fig. 1c), principally at the apical cell surfaces within the biliary canaliculi between the cells, as previously found in the parental PICM-19 cells grown on STO feeder-layers (Talbot et al. 1996). The secretion of serum-proteins by PICM-19FF cells was assessed from a 72 h serum-free CM medium sample. The CM was concentrated

and approximately half of the sample was separated by 2-D gel electrophoresis. As shown in Fig. 1d, the PICM-19FF cells secreted a spectrum of serum-proteins, just as the parental PICM-19 cells did when grown on STO feeder-layers (Talbot et al. 1994a, 2010a) and as primary pig hepatocytes in STO feeder-cell co-culture do (Caperna et al. 2010, 2011). These results further demonstrate that PICM-19FF cells retain the fetal hepatocyte functions present in the feeder-dependent parental PICM-19 cells.

Discussion

It is critical that hepatocyte cell lines or cultures are defined as much as possible as to their cellular, substrate, and medium constituents for applications in toxicology, pharmacology, artificial liver devices, in vitro models of genetic and infectious liver diseases, and for various other biotechnology and research initiatives. The isolation of a PICM-19 cell population capable of indefinite growth without direct contact with feeder-cells, and which maintain their hepatocyte specific morphology and functions, is therefore a significant step forward in devising a completely defined culture environment for the PICM-19 cell line's growth and maintenance.

The study demonstrates that over extensive continuous culture. The PICM-19FF cells maintained typical hepatocyte morphology and hepatocyte functions in comparison to the parental feeder-dependent PICM-19 cells. The PICM-19FF cells displayed and maintained the ultrastructural features typical of hepatocytes, and they maintained P450 EROD activity, serum-protein production, GGT expression in their biliary canalicular structures, and responsiveness to cAMP stimulation—causing fluid transport into their biliary canaliculi with in vivo-like kinetics, i.e., within minutes. Although it appears that the specific activity of urea production of the PICM-19FF cells decreased over passage, there was a significant difference ($p < 0.01$) in cell material in these later passage cultures such that, on a per ml or per flask basis, the levels of urea, and presumably ammonia, were similar. This observation would suggest that the PICM-19FF cells have adjusted urea production to maintain steady-state medium levels of ammonia associated with the handling of the products of protein turnover (basal) or following the addition of supraphysiological

levels of NH_4Cl . This may be similar to the body's primary ammonia homeostatic mechanism where tight control over ammonia level is maintained by hepatic urea and glutamine synthesis (Walker 2009).

It will now be more straightforward to genetically engineer PICM-19FF cells with the use of selectable markers in order to manipulate and examine various hepatocyte functions at the molecular level. Finally, and as previously discussed (Talbot et al. 2010b), the interpretation of experimental results using the feeder-free PICM-19 cells is greatly simplified since the confounding complexity of the presence of feeder-cells in the culture is absent.

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