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Development, characterization, conservation and storage of fish cell lines: a review

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Abstract Cell lines provide an important biological tool for carrying out investigations into physiology, virology, toxicology, carcinogenesis and transgenics. Teleost fish cell lines have been developed from a broad range of tissues such as ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain, skin. One hundred and twenty-four new fish cell lines from different fish species ranging from grouper to eel have been reported since the last review by Fryer and Lannan (J Tissue Culture Methods 16: 87–94, 1994). Among the cell lines listed, more than 60% were established from species from Asia, which contributes more than 80% of total fish production. This includes 59 cell lines from 19 freshwater, 54 from 22 marine and 11 from 3 brackish water fishes. Presently, about 283 cell lines have been established from finfish around the world. In addition to the listing and a scientific update on new cell lines, the importance of authentication, applications, cross-contamination and implications of overpassaged cell lines has also been discussed in this comprehensive review. The authors feel that the review will serve an updated database for beginners and established researchers in the field of fish cell line research and development.

Keywords Fish · Cell lines · Development · Characterization · Conservation

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Introduction

Fish comprise 48% of the known vertebrate species (Altman and Dittmer 1972), which represents an enormous resource for the development of vertebrate cell and tissue models for use in biomedical sciences. The physiology and blood plasma constituents of teleost fish are much like those of terrestrial vertebrates; therefore, the methodology for culture of cells is also similar. Nevertheless, fish cell culture differs somewhat from mammalian cell culture in having a wider temperature range for incubation. Also, osmolality must be adjusted upward for fish of marine origin. Because of lower metabolic rates than eurythermic cells, fish cells can be maintained with little care for long periods of time. Thus, permanent fish cell lines, in contrast to the mammalian cells, are easier to maintain and manipulate, and unlike primary cultures, produce highly reproducible results (Wolf and Quimby 1976).

Early cultures of primary cells may represent a more appropriate model of tissues in vivo (Freshney 2005). The production of short-term primary cultures, however, suffers from a lack of reproducibility in the initiation, and homogeneity of cultures that limit their application (Bols et al. 1994). Established cell lines are typically derived from malignant tumors, or through spontaneous transformation, or through oncogenic immortalization and such changes bring about continuously proliferating (immortal) cell lines (Freshney 2005).

The rainbow trout, *Salmo gairdneri* gonadal cell line, RTG-2 developed by Wolf and Quimby (1962) was the first permanent cell line of fish origin. Since then many more cell lines have been established. The first review of all fish cell and tissue culture was compiled by Wolf and Mann (1980). A comprehensive global list of freshwater and marine fish cell lines was last published in 1994 by Fryer and Lannan and reported some 159 fish cell lines, established from 74 species or hybrids representing 34 families of fish. We have reviewed the research work carried out since this review and report 124 new established cell lines during this period. Among the cell lines listed, more than 60% were established from Asian region, which contributes more than 80% of total fish production. This include 59 cell lines from 19 freshwater, (Table 1), 53 from 22 marine (Table 2) and 11 from 3 brackish water (Table 3) fishes.

Establishment of cell lines from different tissues

Most fish cell lines originated from normal tissues viz., skin, gill, heart, liver, kidney, spleen, swim bladder, brain, etc. Particularly, embryos or fins are most frequently listed as the source of the tissues used in the primary culture. After ovary, the second most common tissue used for cultivation is fin, due to its high regenerative ability (Fryer and Lannan 1994). Surprisingly, there are not a high number of cell lines originating from gonadal or ovarian tissues since these tissues would also exhibit high levels of mitosis. Cell lines have also been developed from ovary (Kumar et al. 2001), skin and fin (Lakra and Bhonde 1996), vertebrae (Pombinho et al. 2004), scales (Akimoto et al. 2000), etc. However, only one cell line XM (Barnes et al. 2006) was initiated from skin and fin tissue of fish melanoma; and in some cases, these cells remained tumorogenic in vivo following repeated in vitro passage.

Many fish cell lines have been established from fish tissues for the purpose of detection and isolation of fish viruses. The cell lines from different tissues of different species will be valuable for studying species-specific responses to viral infection at the cellular level. Some pathogenic viruses are known to be organ- or tissue-specific, which makes the establishment of additional cell lines from different organs and tissues of a host species essential for proper monitoring of viral diseases.

Embryonic and larval cells are the most easy to cultivate being mitotically activate. In the past, it was difficult to obtain eggs or fry of some fish species because they are pelagic spawners (Wolf and Quimby 1966). However, due to recent advances, many species, which were previously unavailable in embryonic form, are now routinely cultivated for the aquaculture industry. In recent years, a number of embryonic stem-like cells were established by various workers from fish species. To develop embryonic stem (ES) cell lines and gene targeting technique in fish, extensive studies have been done in small model fishes, such as zebra fish (*Danio rerio*) and medaka (*Oryzias latipes*), because they offer the possibility of combining embryological, genetic and molecular analysis of vertebrate development. The ES-like cell lines have been established in medaka (Hong et al. 1996, 2000) and zebrafish (Sun et al. 1995). A pluripotent cell line, LJESl, has been established from blastula-stage embryos of *Lateolabrax japonicus,* and these cells differentiated into different types of cells after retinoic acid treatment (Chen et al. 2003a). A continuous embryonic (SISE) cell line has been established from blastula-stage embryos of sea bass (*Lates calcarifer*) (Parameswaran et al. 2006b) and a pluripotent embryonic stem cell line SBES from blastula-stage embryos of sea bass (*Lates calcarifer*) (Parameswaran et al. 2006c) and catfish (*Heteropneustes fossilis*) (Lakra 2010).

Media and additives

Nowadays, the commercialization of technology like ready to use sterile plastic wares and tissue culture media, enzyme solutions, other reagents could overcome the problems in the media preparations like pH, osmolality, sterility, anchorage of cells, nutrients. In

Table 1 Details of cell lines developed from different freshwater fishes from 1994 to 2010

S. no.	Designation	Species	Tissue	Morphology of cells	Medium	Incubation	Passage	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
1	RBTE 45	Rainbow trout *Oncorhynchus mykiss*	Embryo	NM	RPMI 1640 with 10% FBS	20°C	32	56	NM	NM	Susceptible to three strains of IHNV namely HV 90, 220-90 and Elk River	Ristow and De Avila (1994) USA
2	RT-gill W1	Rainbow trout *Oncorhynchus mykiss*	Gill	Polygonal or epithelial-like	Leibovitz L-15 medium with 5–10% FBS	NM	50	NM	Contaminated with mycoplasma, but eradicated	NM	The cell line supports the replication of ISAV but CPE is not observed	Bols et al. (1994) Canada
3	–	Rainbow trout *Oncorhynchus mykiss*	Liver	Epithelial	EMEM 10% FCS	20°C	NM	NM	NM	NM	NM	Ostrander et al. (1995) USA
4	TPS	Rainbow trout *Oncorhynchus mykiss*	Pronephos	Fibroblastic, epithelioid and giant	RPMI-1640 with 10% FCS	18–22°C	104	58	Cytochemistry, electron microscopy isoenzyme profile	NM	Susceptible to VHSV and IHNV	Diago et al. (1995) Spain
5	MG-3	Mrigal *Cirrhinus mrigala*	Gill	Fibroblast-like	Leibovitz L-15 medium with 10% FCS	28°C	20	50	Profile of three isoenzymes	Cryopreserved and revived	NM	Sathe et al. (1995) India
6	Macrophage cell line	Goldfish *Carassius* sp.	Kidney	Macrophage	Leukocyte medium 10% FCS and 5% goldfish serum		50	100	NM	NM	NM	Wang et al. (1995) Canada
7	EP-1	Japanese eel Elver *Anguilla japonica*	Viscera	Giant squamous cells and fibroblastic	Leibovitz L-15 medium with 10%FCS	28°C	NM	32	Persistently infected with *Plesitophora anguillarum*	NM	NM	Kou et al. (1995) Taiwan
8	RG-1	Rohu *Labeo rohita*	Gill	Fibroblast-like	Leibovitz L-15 medium with 10% FCS	28°C	20	50	Profile of three isoenzymes	Cryopreserved and revived	NM	Sathe et al. (1997) India
9	GFSk-S1	Goldfish *Carassius auratus*	Skin	Epithelial and fibroblastic	Leibovitz L-15 medium with 10% FBS	25–28°C	Routinely for 5 years	NM	NM	Cryopreserved and revived	NM	Lee et al. (1997) Canada
10	PSP	Barb *Puntius schwanenfeldi*	Fin	Epithelial-like	Leibovitz L-15 medium with 20% FBS	28°C	32	NM	NM	Cryopreserved and revived	Susceptible to IPNV and rhabdovirus	Karunasagr et al. (1998) India
11	RTS11	Rainbow trout *Oncorhynchus mykiss*	Spleen	Macrophage-like	Leibovitz L-15 medium 20–30% FBS	18°C	Maintained for over 1 year	NM	NM	Not able to revive	NM	Ganassin and Bols (1998) Canada

Table 1 continued

S. no.	Designation	Species	Tissue	Morphology of cells	Medium	Incubation	Passage	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
12	5C2	Rainbow trout *Oncorhynchus mykiss* Hot Creek 71 strain	Embryo	Epithelial-like	RPMI-1640 with 10% FBS	20°C	NM	58	NM	Restriction fragment length polymorphisms	Susceptible to IHNV	Ristow and Thorgaard (1998) USA
13	6B	*Oncorhynchus mykiss* Hot Creek 71 strain		Epithelial-like				60				
14	11B	*Oncorhynchus mykiss* Arlee 12 Strain		Fibroblast-like				64				
15	23B2	*Oncorhynchus mykiss* Arlee 12 Strain		Fibroblast-like				71				
16	24BC	*Oncorhynchus mykiss* Arlee 12 Strain		Epithelial-like				65				
17	25B	*Oncorhynchus mykiss* Arlee 12 Strain		Epithelial-like				59				
18	26B	*Oncorhynchus mykiss* Arlee 12 Strain		Epithelial-like				67				
19	58B2C	*Oncorhynchus mykiss* OSU 142		Epithelial-like cells				64				
20	49B2	*Oncorhynchus mykiss* OSU 142		Epithelial-like				62				
21	–	Rainbow trout *Oncorhynchus mykiss*	Spleen	Multilayers of epithelioid and fibroblastic	RPMI-1640	19°C	NM	NM	Cytochemical and immuno-cytochemical analyses, phagocytic activity	NM	Susceptible to VHSV	Flano et al. (1998) Spain
22–27	A-6, B-7, E-2, E-9, E-11 and C-3	Snake head *Ophicepalus striatus*	Fry (Clone of SSN-1)	B-7 and E-11 were round-shaped and the others were spindle-shaped	Leibovitz L-15 medium with 10% FBS	A-6, B-7, E-2, E-9 and E-11–25 and 20°C	NM	NM	NM	NM	Susceptible to 4 piscine nodavirus strains	Iwamoto et al. (2000) Japan

Table 1 continued

S. no.	Designation	Species	Tissue	Morphology of cells	Medium	Incubation	Passage	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
28	GAKS	Goldfish *Carassius auratus*	Scales	Epithelial type	DM 160 10% FBS	NM	NM	NM	Contained alkaline phosphatase activity and secreted endothelin	NM	NM	Akimoto et al. (2000) Japan
29	–	African catfish *Clarias gariepinus*	Ovary	NM	Leibovitz L-15 medium as the base, with FBS 10%, fish muscle extract 10%, prawn muscle extract 10%, concanavalin A. 0.02 mg, lipopolysaccharide 0.02 mg, glucose D 0.2 mg, Ovary extracts 0.5% prawn hemolymph 0.5%	26 ± 28°C	15	NM	NM	NM	NM	Kumar et al. (2001) India
30 31 32	SHMS SHHT SHSB	Snakehead *Channa striatus*	Muscle Heart Swim bladder	NM	Leibovitz L-15 medium 20% FBS RPMI 1640	25°C	13–18	44	NM	Survival rate 95.9–96.6%	Susceptible to VHSV, SVCV, IPNV and SHRV	Zhao et al. (2003) USA
33	WBE	White bass *Morone chrysops*	Embryo	Epithelial	DMEM or Lebovitz L-15 medium 10% FBS	31°C	80	$2n = 44$ or $2n = 49$	Positive immunohistochemical staining for cytokeratin and intercellular junctions when observed by electron microscopy	Survival rate 90%	NM	Himizu et al. (2003) USA
34 35 36	WSF WSHST WSBM	White sturgeon *Acipenser transmontanus*	Fin Head soft tissue Body muscle	Spindle-shaped	Leibovitz L-15 medium 20% FBS	15–30°C	35	NM	Sequence analysis of 16S rRNA	Survival rate 90%	NM	Wang et al. (2003) USA
37	RTHDF	Rainbow trout *Oncorhynchus mykiss*	Skin	Fibroblasts long bipolar	Leibovitz L-15 medium with 20% FBS	21°C	34	NM	Telomerase activity was demonstrated early and late studying biochemical responses of teleost cells to environmental stress	Cryopreserved at passages 3, 7, 11, 28 and 34	NM	Ossum et al. (2004) Denmark

Table 1 continued

S. no.	Designation	Species	Tissue	Morphology of cells	Medium	Incubation	Passage	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
38	XM	*Xiphophorus* BC1 hybrids (*Xiphophorus maculatus_ Xiphophorus helleri_ Xiphophorus helleri*	Melanoma	NM	50% DMEM, 35% L-15 and 15% Ham's F-12, sodium bicarbonate and HEPES buffer	24°C	NM		NM	NM	NM	Baranes et al. (2006) USA
39	TP-1	Golden mahseer *Tor putitora*	Fry	Fibroblastic	Leibovitz L-15 with 20% FCS and 10% FME	28°C	20	100	Cell cycle analysis	Survival rate 70%	NM	Lakra et al. (2006b) India
40	PBLE	American eel, *Anguilla rostrata*	Leukocytes	Fibroblastic	Leibovitz L-15 medium with 20% FBS	18°C	80	38	Cells underwent apoptosis in response to gliotoxin, but did not show a respiratory burst	NM	Susceptible to CSRV	Dewitte-orr et al. (2006) Canada
41	GFM	*Goldfish Carassius auratus*	Muscle	Epithelial	Leibovitz L-15 medium with 20% FBS	30°C	22	104	Sequence analysis of 16S and 18S rRNA	Survival rate 93%	Susceptible to IHNV, IPNV, CSRV, SVCV	Luc Rougée et al. (2007)
42	GFSB		Swim bladder	Fibroblastic		30°C	22	104	Sequence analysis of 16S and 18S rRNA	Survival rate 92%		USA
43	–	Siberian sturgeon *Acipenser baerii*	Head kidney	Polynucleated	DMEM 20% FCS		24		Cytochemistry Co-imunostain with cross-reactive antibodies	NM	NM	Ciba et al. (2008) Germany
44	SICE	Catla *Catla catla*	Eye tissue	Epithelial-like	Leibovitz L-15 with 15% FBS	28°C	80	50	Sequence analysis of 12S rRNA, Transfected with pEGFP vector	Survival rate 80%	Susceptible to six marine fish viruses, viz., VNNV, MABV-NC1, CSRV, IHNV, IPNV and hirame rhabdovirus	Ahmed et al. (2008) India
45	CSTF	Chinese sturgeon *Acipenser sinensis*	Fin	Epithelial	DMEM	25°C	NM	$2n = 264$	Transfected with pEGFP vector DNA	NM	Susceptible to RGV	Zhou et al. (2008) China
46	MFF-1	Mandarin fish *Siniperca chuatsi*	Fry	Epithelial-like	DMEM with 10% FBS	27°C	60	32, 36 and 48	NM	Survival rate 80–90%	Susceptible to Infectious spleen and kidney necrosis virus	Dong et al. (2008) China

Table 1 continued

S. no.	Designation	Species	Tissue	Morphology of cells	Medium	Incubation	Passage	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
47	SICH	Catla *Catla catla*	Heart	Fibroblast-like	Leibovitz L-15 with 15% FBS	28°C	130	NM	Sequence analysis of 12 s rRNA, Transfected with pEGFP vector	Survival rate 85–90%	ECP from *Aeromonas* sp., or *Vibrio anguillarum* were found to be toxic	Ahmed et al. (2009a) India
48	RE	Rohu *Labeo rohita*	Eye	Epithelial	Leibovitz L-15 with 20% FBS	28°C	70	NM	Sequence analysis of 12S rRNA, transfected with pEGFP	Survival rate 80%	ECP from *Aeromonas* sp. were toxic to the cell lines	Ahmed et al. (2009b) India
49	CB	Catla *Catla catla*	Brain	Fibroblast-like	Leibovitz L-15 with 20% FBS	28°C	35	NM				
50	mRTP1B	Rainbow trout *Oncorhynchus mykiss*	Pituitary glands	NM	CO_2-independent medium with 10% FBS	20°C	150	60 ± 2	Expressed other pituitary-specific genes Immunocytochemical analysis showed that all the five single-cell clones produced both Gh and Prl.	NM	NM	Chen et al. (2010) USA
51	mRTP1E											
52	mRTP1F											
53	mRTP1K											
54	mRTP2A											
55	RH	Rohu *Labeo rohita*	Heart	Long fibroblast-like cells	Leibovitz L-15 medium with 10% FBS	28°C	NM	NM	Sequence analysis of 16S rRNA and COI	Survival rate 75%	NM	Lakra et al. (2010)
56	RF		Fin	Fibroblast-like cells						Survival rate 70%		
57	RSB		Swim bladder	Epithelial-like cells						Survival rate 72%		
58	PSF	Pearl spot *Etroplus suratensis*	Caudal fin	Epithelial cells	Leibovitz L-15 with 15% FBS	28°C	35	$2n = 48$	16sS rRNA and COI sequencing	Survival rate 70%	ECP from *Vibrio cholerae* MTCC 3904 were found to be toxic	Swaminathan et al. (2010)

bFGF, Basic fibroblastic growth factor; COI, cytochrome oxidase subunit I; CPE, cytopathic effect; CSRV, chum salmon reovirus; DMEM, Dulbecco's minimum essential medium; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; FCS, fetal calf serum; GRP, green fluorescent protein; IHNV, infectious hematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; ISAV, infectious salmon anemia virus; MABV-NC1, marine birnavirus NC1; NM, not mentioned; RGV, *Rana grylio* virus; SHRV, snakehead rhabdovirus; SVCV, spring viremia carp virus; VHSV, viral hemorrhagic septicemia virus; VNNV, viral nervous necrosis virus

Table 2 Details of cell lines developed from different marine fishes from 1994 to 2010

S. no.	Designation	Species	Tissue	Morphology	Medium	Incubation	Passage level	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
1	CoE 345	Coho salmon *Oncorhynchus kisutch*	Embryo	NM	RPMI 1640 with 10% FBS	20°C	34	59	NM	NM	Susceptible to three strains of IHNV namely HV 90, 220-90 and Elk River	Ristow and De Avila (1994) USA
2	CoE 45						36	60				
3	CoE 115						43	60				
4	SHK-1	Atlantic salmon *Salmo salar*	Head kidney	Fibroblast-like	Leibovitz L-15 medium with FCS 5%	15–20°C	30	NM	NM	NM	Susceptible to ISAV	Dannevig et al. (1995) Norway
5	SLW	Spot Croaker *Leiostomus xanthurus*	Liver	Hepatocyte	RPMI-1640 and L-15 with 10% FBS	21–27°C	NM	48	Macromelcular analysis was done, response to mitogens like coral tree extract, LPS and Concovalin A was analyzed	NM	NM	Faisal et al. (1995) USA
6	SLN			Stellate in shape				48				
7	SLF			Spindle shape				Not done				
8	FG-9307	Flounder *Paralichthys olivaceus*	Gill	Epithelioid	Leibovitz L-15 medium with 20% FBS	20–25°C	96	NM	NM	NM	Not susceptible to IPNV	Tong et al. (1997) China
9	SAF-1	Gilt-head seabream *Sparus aurata*	Fin	Fibroblast-like	DMEM/Nutrient mixture Ham,s F12 with 5% FBS	25°C	70	48	NM	Cryopreserved and thawed	Susceptible to rhabdoviruses and *Vibrio* ECP	Bejar et al. (1997) Spain
10	SPH	Sea perch *Lateolabrax japonicus*	Heart	Epithelioid	EMEM with 10% FBS	25°C	106	48	NM	NM	Susceptible to IPNV	Tong et al. (1998)
11	SPS		Spleen	Epithelioid	EMEM with 10% FBS	25°C	102					China
12	RSBF	Red sea bream *Pagrosomus major*	Fin	Fibroblast-like cells	EMEM with 10% FBS	30°C	96					
13	GF-1	Orange-spotted grouper *Epinephelus coioides*	Fin	Fibroblastic together with epithelioid	Leibovitz L-15 medium with 5% FBS	28°C	120	32 and 36	NM	Survival rate after 1 year is 73%	Susceptible to IPNV, HCRV, Eel herpes virus, GNNV	Chi et al.(1999) Taiwan
14	GK	Yellow grouper *Epinephelus awoara*	Kidney	Fibroblastic and epithelial	Leibovitz L-15 Medium with 10% FBS	28°C	120	60	NM	Survival rate 95%	Susceptible to GIV	Lai et al. (2000)
15	GL		Liver					76				Taiwan
16	ASK	Sea trout *Salmo trutta*	Head kidney	NM	Leibovitz L-15 medium with 15% FCS	15–20°C	30	NM	NM	NM	Susceptible to ISAV	Devold et al. (2000) Norway

Table 2 continued

S. no.	Designation	Species	Tissue	Morphology	Medium	Incubation	Passage level	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
17	TO	Atlantic Salmon *Salmo salar*	Head kidney	NM	E MEM with Hanks' BSS (HMEM Leibovitz L-15 medium	20°C	150		NM	NM	Susceptible to ISAV	Wergeland and Jakobsen (2001) Norway
18	GB	Yellow grouper *Epinephelus awoara*	Brain	Fibroblast-like and epithelioid	Leibovitz L-15 medium with 10% FBS	24 and 32°C	80	22 ± 42	NM	NM	Susceptible to YGNNV	Lai et al. (2001) Taiwan
19	GSB	Yellow grouper *Epinephelus awoara*	Swim bladder	Epithelial-like	Leibovitz L-15 medium with 10% FBS	28°C	81	86	Transfected with pEGFP-1, pEGFP-C1, pEGFP-aMT2.5	Survival rate 95%	Susceptible to YGNNV and GIV	Lai et al. (2003) Taiwan
20	GH		Heart	Fibroblast-like				58				
21	GE		Eye	Epithelial-like				48				
22	GF		Fin	Fibroblast-like cells				66				
23	FSP	Japanese flounder *Paralichthys olivaceus*	Spleen	Epithelioid cells	EMEM with 10% FBS	20°C	100	62	Analysis of the microsatellite markers		Susceptible to IPNV, MABV-NV1, CSV, IHNV, SVCV, Hirame rhabdovirus	Kang et al. (2003) Korea
24	FFN		Fin	Epithelioid cells				64				
25	PL	Japanese Plichard *Sardinops sagax neopilchardus*	Liver	Epithelial-like	EMEM 10% FBS	20–22°C	80	48 at 10th passage 38–102 at 57th passage	Sequence analysis of the mitochondrial DNA	LN_2 and revived	Susceptible to IPNV (Sp strain), IHNV (WRAC strain), VHSV (strain 23.75), EHNV, SVCV and ASRV	Williams et al. (2003) Australia
26	PH		Heart				40					
27	LJES1	Sea perch *Lateolabrax japonicus*	Embryo	Small and round or polygonal	DMEM with 15% FBS, marine fish serum, sea perch embryo extract, selenium, bFGF and leukemia inhibitory factor	25°C	40	48	When the cells were treated with all-trans retinoic acid, differentiation into various types of cells	Survival rate 60%		Chen et al. (2003a) China
28	WSF	White sturgeon *Acipenser transmontanus*	Fin	Spindle-shaped	Leibovitz L-15 medium 20% FBS	15–30°C	35	NM	Sequence analysis of 16S rRNA	Survival rate 90%	NM	Wang et al. (2003) USA
29	WSHST		Head soft tissue									
30	WSBM		Body muscle									

Table 2 continued

S. no.	Designation	Species	Tissue	Morphology	Medium	Incubation	Passage level	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
31	SBES1	Red sea bream *Chrysophrys major*	Embryo	NM	DMEM medium with 15% FBS, marine fish serum, fish embryo extract, selenium, Bfgf and LIF	24°C without CO_2	60	2n¼2stþ 46t	Treated with all-trans retinoic acid, they differentiated into various types	NM	NM	Chen et al. (2003b) China
32	FEC	Japanese flounder *Paralichthys olivaceus*	Embryo	Epithelial-like cells	DMEM 15% FBS, seaperch serum and bFGF	24°C in an ambient air incubator	60	$2n = 48$	NM	Survival rate 60%.	Susceptible to 2 fish iridoviruses	Chen et al. (2004) China
33	RGF	Atlantic salmon *Salmo salar*	Gill	Fibroblastic	Leibovitz L-15 medium, with 15%FBS	22°C	70	NM	Reacted with MAbs against mammalian fibronectin and type I collagen	NM	NM	Butler and Nowak (2004) Australia
34	RGE-2	Atlantic salmon *Salmo salar*	Gill	Epithelial	Leibovitz L-15 medium, with 10% FBS		68		Reacted with MAbs against mammalian cytokeratins			
35 36	VSa13 VSa16	Gilthead sea bream *Sparus aurata*	Vertebra	Spindle-like	Leibovitz L-15 with 10% FBS	22°C	35		Analyzing the expression and regulation of cartilage—and bone-specific genes	NM	NM	Pombinho et al. (2004) Portugal
37	TEC	Turbot *Scophthalmus maximus*	Gastrula	Small and round	DMEM 15% FBS, sea perch serum and bFGF	24°C	60	$2n = 4m + 12st + 28t$	NM	NM	NM	Chen et al. (2005) China
38 39	BTMS BTF	Blue fin Trevally *Caranx melampygus*	Muscle Fins	Fibroblast-like	Leibovitz L-15 medium with 20% FBS and also well in RPMI 1640, M199 and MEM	25°C	37 41	48	NM	Survival rate 98%	Sensitive to IHNV, IPNV, SVCV, VHSV and SHRV	Zhao and Lu (2006) USA
40	HEW	Haddock *Melanogrammus aeglefinus*	Embryos	Epithelial-like	Leibovitz L-15 medium with 15% FBS	12–18°C	47	66	Sequencing two housekeeping genes	NM	NM	Bryson et al. (2006) Canada
41	SIGE	Orange-spotted grouper *Epinephelus coioides*	Eye	Epithelial	Leibovitz L-15 medium with 15% FBS	28°C	100	48	NM	Survival rate 90%	Susceptible to Nodavirus, MABV NC-1 and Y6	Parameswaran et al. (2007) India

Table 2 continued

S. no.	Designation	Species	Tissue	Morphology	Medium	Incubation	Passage level	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
42	CRF-1	Red sea bream, *Pagrus major*	Fin	Fibroblast	DMEM nutrient mixture F12 HAM growth medium with 20% FBS	24°C	NM	48	NM	NM	Used to study the phylogenetic relationships of a recent RSIV isolate	Imajoh et al. (2007) Japan
43	GSC	Red-spotted grouper *Epinephelus akaara*	Snout	Fibroblast	DMEM with 10% FBS	25°C	60	$2n = 8st + 40t$	NM	Transfected with pEGFP vector	Susceptible to SMRV	Zhou et al. (2007) China
44	GBC1	Orange-spotted grouper *Epinephelus coioides*	Brain	Epithelial cells	Leibovitz L-15 medium with 5% FBS	20 and 35°C	NM	44	NM	NM	Susceptible to GNNV	Wen et al. (2008) Taiwan
45	GBC4				Leibovitz L-15 medium with 10% FBS			48	GBC4 cells expressed glial fibril acidic protein suggesting that they are astroglial lineage cells	NM	Susceptible to the GSIV	
46	RGB	Rockfish grouper *Epinephelus quoyanus*	Brain	Epithelioid	Leibovitz L-15 medium with 10% FBS	30°C	39	48	Transfected with pEGFP-C3. Analysis of cytochrome b gene sequences	NM	Susceptible to HCRV and GNNV	Ku et al. (2009) Taiwan
47	RGG		Gill	Epithelioid			45				NM	
48	RGH		Heart	Fibroblastic			35				Susceptible to GNNV	
49	EAGS	Red-Spotted Grouper *Epinephelus akaara*	Spleen	Fibroblastic	Leibovitz L-15 medium with 10% FBS	25 and 30°C	NM	88	NM	NM	Susceptible to SGIV	Huang et al. (2009) China
50	EAGSB		Swim bladder	Epithelioid				72				
51	CSEC	Half smooth tongue sole *Cynoglossus semilaevis*	Embryo	NM	DMEM with 15% FBS bFGF LIF and mercaptoethanol	24°C	50	$2n = 42t$	Transfected with the GFP reporter gene	NM	Susceptible to LCDV	Sha et al. (2010) China
52	CSH	Half smooth tongue sole *Cynoglossus semilaevis*	Heart	Elongated fibroblastic	MEM with 10% FBS and 2 ng/ml bFGF	24°C	60	$2n = 42$	Transfected with the GFP reporter gene	Survival rate 60–70%	Susceptible to LCDV	Wang et al. (2010a) China
53	Cod ESC	Atlantic cod *Gadus morhua*	Embryo	NM	DMEM with 4.5 g/l glucose and 25 mM HEPES, with 10% FCS and other nutrients	17°C	NM	NM	Express a class V POU transcription factor, designated ac-Pou2	NM	NM	Holen et al. (2010) Norway

Table 2 continued

S. no.	Designation	Species	Tissue	Morphology	Medium	Incubation	Passage level	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
54	TK	Turbot *Scophthalmus maximus*	Kidney	Fibroblast-like	MEM with HEPES, FBS, 2-mercaptoethanol, bFGF	24°C	50	$2n = 44$	Transfected with pEGFP-N3 vector	NM	Susceptible to TRBIV	Wang et al. (2010b) China

ASRV, Atlantic salmon reovirus; CSV, chum salmon virus; DMEM, Dulbecco's minimum essential medium; EHNV, epizootic haematopoietic necrosis virus; EMEM, Eagle's minimum essential medium; GIV, grouper iridovirus; GNNV, grouper nerve necrosis virus; GSIV, giant seaperch iridovirus; HCRV, hard clam reovirus; IHNV, infectious hematopoietic necrosis virus; IPNV, infectious pancreatic necrosis virus; ISAV, infectious salmon anemia virus; MABV-NC1, marine birnavirus NC1; LCDV, lymphocystis disease virus; LIF, leukemia inhibitory factor; MAb, monoclonal antibody; NM, not mentioned; RSIV, red seabream iridovirus; SGIV, Singapore grouper iridovirus; SHRV, snakehead rhabdovirus; SMRV, *Scophthalmus maximus* rhabdovirus; SVCV, spring viremia carp virus: TRBIV, turbot reddish body iridovirus; VHSV, viral hemorrhagic septicemia virus; YGNNV, yellow grouper nervous necrosis virus

general, most fish cultures use media developed for mammalian cell culture. Eagle's Minimal Essential Medium (EMEM) supplemented with fetal bovine serum (FBS) comes close to being an all purpose culture medium for the cells of mammals, birds, reptiles, amphibians and of course fish (Wolf and Quimby 1966). Other media routinely used in fish culture are Glasgow MEM, Hank's MEM (HMEM) and Leibovitz L-15 medium (L-15). An amino acid-rich nutrient medium such as L-15 that does not require CO_2 buffering (Leibovitz 1963) has been successfully used with fish cell lines, thus CO_2 incubators are not necessary, and cells can be grown conveniently in any undisturbed areas. Due to this advantage, more than 80% of the cell lines established after 1994 used Leibovitz L-15 media. However, some primary cell lines have had specific culture medium designed to optimize growth during development of the primary culture (Wang et al. 1995).

Fetal bovine serum (FBS) seems to be the most popular choice of supplements with the tissue culture media as it is easy to obtain in large volumes and due to the presence of known and unknown growth factors. Wolf and Quimby (1966) quoted instances in which other types of serum have been used but the results appeared to be of mixed benefits. Fish serum was used (<1%) in combination with FBS in developing fish cell lines (Chen et al. 2004; Lakra et al. 2006a). Serum concentration can also have an effect on primary cultures. Throughout the literature, concentration varies from 5% to as high as 20%. Serum concentrations are not usually much higher than this, as there is evidence that high serum concentrations may inhibit cell growth (Freshney 2005).

Most examples of additives to media are in serum-free or reduced media replacing substances that serum would normally provide (Wang et al. 1995). However, that does not entirely discount the possibility of using further additives to media already supplemented with serum. Kumar et al. (2001) used a large list of additives for example, fish muscle extract, sucrose, prawn shell extract, which were explored during developing a primary culture from ovary tissue of African catfish.

Miller et al. (1994) detailed the use of chemical mitogens used to establish suspension cultures from leukocytes. Cell cultures exhibited a strong proliferative response after exposure to the mitogens. Faisal

Table 3 Details of cell lines developed from different brackish water fishes from 1994 to 2010

S. no.	Designation	Species	Tissue	Morphology	Medium	Incubation (°C)	Passage level	Karyotype	Characterization	Cryopreservation	Virus susceptibility/ application	Reference/ country
1	SF	Asian sea bass *Lates calcarifer*	Fry	Epithelial-like	EMEM with 15% FCS	25	80	NM	NM	Survival rate 85%	Susceptible to iridoviruses, birnaviruses, reoviruses, a rhabdovirus, and a nodavirus	Chang et al. (2001) Singapore
2	SISK	Asian sea bass *Lates calcarifer*	Kidney	Epithelial-like	Leibovitz L-15 medium with 15% FBS	28	100	48	Positive for epithelial cells markers	Survival rate was 80–90%	Susceptible to MABV NC1 and nodavirus	Hameed et al. (2006) India
3	SISE	Asian sea bass *Lates calcarifer*	Blastula	Epithelioid	Leibovitz L-15 medium with 15% FBS	28	70	48	Characterized by CFLSM, transfection with pEGFP-N1, proliferate marker (BrdU)	Survival rate was 70–80%	Susceptible to IPNV VR-299 and nodavirus	Parameswaran et al. (2006a) India
4	SBES	Asian sea bass *Lates calcarifer*	Blastula	Epithelioid	Leibovitz L-15 medium with 15% FBS	28	70	48	On treatment with all-trans etinoic acid, these cells differentiated into neuron-like cells, muscle cells and beating cardiomyocyte	NM	Susceptible to nodavirus and IPNV VR299	
5	SISS	Asian sea bass *Lates calcarifer*	Spleen	Fibroblast and epithelial-like	Leibovitz L-15 medium with 15% FBS	28	70	NM	Immunocytochemistry, CFLSM, transfected with pEGFP-N1	Survival rate 70–80%	Susceptible to IPNV VR-299 and nodavirus	Parameswaran et al. (2006b) India
6	LCE	Asian seabass *Lates calcarifer*	Fry	Epithelioid	Leibovitz L-15 medium with 20% FBS	28	15	NM	NM	Survival rate 70%	NM	Lakra et al. (2006a) India
7	LCF		Fry	Fibroblastic								
8	SIMH	Milkfish *Chanos chanos*	Heart	Fibroblast-like	Leibovitz L-15 medium with 20% FBS	28	50	NA	Transfected with pEGFP-N1 immunocytochemistry	Survival rate 90%	Not susceptible to seven fish viruses	Parameswaran et al. (2007) India
9	BM	Barramundi *Lates calcarifer*	Muscle	Fibroblastic	Leibovitz's L-15 medium with 10% FBS	28	100	NM	The induction of apoptosis was assayed infected cells	NM	Susceptible to GIV	Lai et al. (2008) Taiwan
10	BSB	Barramundi *Lates calcarifer*	Swim bladder	Epithelial								
11	SBB-W1	European sea bass *Dicentrarchus labrax*	Brain	Neuronal	Leibovitz L-15 medium with 10% FBS	22	23	NM	Immunostaining with a glial and a neuronal marker	NM	NM	Servili et al. (2009) Canada

CFLSM, confocal laser scanning microscopy; DMEM, Dulbecco's minimum essential medium; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; FCS, fetal calf serum; GIV, grouper iridovirus; IPNV, infectious pancreatic necrosis virus; MABV NC1, marine birnavirus NC1; NM, not mentioned

et al. (1995) exposed cultured liver cells to plant-derived mitogens that stimulated DNA synthesis (indicative of cell proliferation). Various growth factors such as mammalian epidermal growth factor (mEGF) (Watanabe et al. 1987), basic fibroblast growth factor (bFGF) (Chen et al. 2004) had been used to stimulate growth of fish cell lines, and bFGF is a potent mitogen for embryonic stem cells derived from *Oryzias latipes* (Hong et al. 1996) and sea perch (Chen et al. 2003b), lymphoid cells from *Penaeus monodon* (Hsu et al. 1995), embryonic cells from *Paralichthys olivaceus* (Chen et al. 2004).

Immortalization of cells

Normal somatic cells have a finite life span and become senescent after a predictable number of cell divisions (Hayflick and Moorehead 1961). Cellular senescence is triggered by two interdependent mechanisms. One induces cell cycle arrest and is controlled by two tumor suppressor pathways, p19ARF/p53 and p16INK4a/Rb (Kiyono et al. 1998). The second is a critical shortening of the telomeres due to the end-replication problem in chromosome replication (Aviv and Harley 2001). It has been documented that a small number of cells arise spontaneously immortalized by a set of genetic alterations. The alterations most frequently observed in immortalized cells are loss of functional Rb (retinoblastoma) or p53 proteins that control two major cell cycle checkpoints (Bodnar et al. 1998). A number of viral oncogenes, including simian virus-40 (SV40) large T-antigen, adenovirus E1A and E1B and polyoma T-antigen, also immortalize cells of a variety of species (Katakura et al. 1998). A catalytic subunit of telomerase ribonucleoprotein (TERT) with reverse transcriptase activity synthesizes and maintains the telomeres, helping cells escape replicative senescence caused by the shortening of telomeres (Bodnar et al. 1998). The preferred method to immortalize cells is through expression of the telomerase reverse transcriptase protein (TERT) (Takakura et al. 1999), particularly those cells most affected by telomere length (e.g., human). Analysis of several telomerase-immortalized cell lines has verified that the cells maintain a stable genotype and retain critical phenotypic markers. A eukaryotic expression plasmid containing the hTERT cDNA is available in ATCC (catalog number ATCC® MBA-141), which will enable researchers to immortalize their own cells.

On the other hand, cell immortalization techniques have attracted enthusiastic attention because they have provided us with cell clones that usually show continual possibility, excellent revitalization after storage and ease of handling in culture. These techniques were mostly used in developing immortal cell lines in humans and other mammalians. But Barker et al. (2000) documented for the first time that the channel catfish long-term leukocyte lines constitutively expressed high levels of telomerase activity. A United States patent (Number. US 6,436,702 B1) was issued for the immortal cell line (spontaneously transformed) derived from grouper *Epinephelus coioides* to Shau-Chi Chi in 2002. In this patent, the monitoring of the transformation of cells, which was characterized by a change in chromosome number distribution, plating efficiency, FBS requirements and the immortalization, was not induced by any methods.

Cross-contamination and over passage

The ease of handling and simple growth requirements make cross-contamination of cell lines a more likely possibility. Fish cell lines are relatively easy to culture, and most have simple growth requirements that make cross-contamination a potential problem. Cell line contamination is not an uncommon incident in laboratories handling more than one cell lines, and many reports have been made on cross-contamination of mammalian cell lines (Parodi et al. 2002). Although problems of misidentification and cross-contamination of fish cell lines have rarely been reported, these are issues of concern for cell culturists that can make scientific results and their reproducibility unreliable. Human cell lines have been reported contaminated with simian cells or murine cells or even other human cells, most ubiquitously, HeLa cells (Tokiwa et al. 1989). Although cross-contamination of fish cells with other cell types has not been widely reported, Perry et al. (2001) conveyed the identification of a cell line dubbed Clone 1A believed to be derived from rainbow trout as being CHSE-214, a cell line derived from Chinook salmon embryos (Lannan et al. 1984). Accordingly, awareness of good laboratory practices and careful vigilance with fish cell cultures as detailed by Lannan

(1994) should be followed to avoid confusion of cell lines. The problem of intraspecies and interspecies cross-contamination among cell lines has been recognized for half a century, and although reviews have been published, evidence of continued use of misidentification and cellular cross-contamination of cell cultures has not declined (Buehring et al. 2004).

In addition, cell lines maintained in culture over a long period of time may experience mutations that alter the original functional characteristics of the cell lines, identified at earlier passage levels (Yu et al. 1997). Furthermore, cell lines do not behave similarly with increased passage number (Hughes et al. 2007). Long-term subculturing places selective pressure on cell line traits leading to, for example, faster growing cells that eventually overrun slower proliferators in the population.

Authentication

When cell lines are obtained from colleagues, they often lack verification or documentation about the condition or passage number of the lines. This practice increases the likelihood that inferior, malperforming cultures are used, leading to results that may not be accurate or reproducible (Wenger et al. 2004). Methodologies for characterizing fish cell lines have included random amplified polymorphic DNA methods (RAPD) (Matsuo et al. 1999) and microsatellite DNA profiling (Perry et al. 2001), mitochondrial 16S and 18S rRNA and sequence analysis (Ahmed et al. 2009a), which has proven useful for identifying a handful of fish cell lines. A simple proteomic approach has been made to identify several fish cell lines derived from tissues of the same or different species. Protein expression signatures (PES) of the evaluated fish cell lines have been developed using 2-DE and image analysis, and it could thus serve as an additional, valuable and reliable technique for the identification of fish cell lines (Wagg and Lee 2005).

Applications

The availability of fish cell lines, since the 1960s, has begun to make impacts in scientific research, but at a much slower rate than with mammalian cell lines. Early work with fish cell lines was initiated with RTG-2, mainly for virological studies (Wolf and Quimby 1962). Fish cell lines are also finding roles in areas with impacts beyond that of the diseases of fish and are providing important contributions in studies relating to toxicology, carcinogenesis, genetic regulation and expression and DNA replication and repair. In the almost 50 year since then, fish cell lines have grown in number covering a wide variety of species and tissues of origin and an array of applications. Fish immunology (Bols et al. 2001), toxicology (Bahich and Borenfreund 1991), ecotoxicology (Schirmer 2006), endocrinology (Bols and Lee 1991), virology (Wolf 1988), biomedical research (Hightower and Renfro 1988), disease control (Villena 2003), biotechnology and aquaculture (Bols and Lee 1991) and radiation biology (Ryan et al. 2008) are some of the areas in which fish cell lines have made significant contributions.

As exogenous DNA delivery of cultured cell is very useful for both basic research and biotechnological applications, it is necessary to determine the transfection efficiency and gene expression on newly developed cell lines. Various workers observed significant fluorescent signals when the cell lines were transfected with pEGFP vector DNA, indicating their potential utility for transgenic and genetic manipulation studies (Qin et al. 2006; Ye et al. 2006; Parameswaran et al. 2007; Zhou et al. 2007; Ahmed et al. 2008; Ku et al. 2009).

A common method for determining whether a virus is present in a healthy fish population is to attempt to isolate it in an appropriate cell line. A cell line will also allow further study of viruses isolated in disease outbreaks. The different cell lines were tested for the susceptibility to various viruses. Lai et al. (2003) found that besides these four cell lines, previously established grouper brain, kidney and liver cell lines were also used for a viral susceptibility study, which showed that all the cell lines were sensitive to grouper iridovirus, whereas only brain, fin and liver cell lines were susceptible to the yellow grouper nervous necrosis virus (a nodavirus). Five fish viruses were tested on this cell line to determine its susceptibility to these viruses and this was found to be susceptible to MABV NC1 and nodavirus, and the infection was confirmed by RT-PCR and CPE. (Hameed et al. 2006). The SIGE cell line was found to be susceptible to nodavirus, MABV NC-1 and Y6, (Parameswaran

et al. 2007). PBLE was susceptible to Chum salmon reovirus (CSV) and supported CSV replication. (Dewitte-Orr et al. 2006). GS cell cultures showed advanced cytopathic effects after infection with a pathogenic grouper iridovirus (Singapore grouper iridovirus, SGIV) or with a grouper nodavirus (*Epinephelus tauvina* nervous necrosis virus, ETNNV) (Qin et al. 2006). GBC4 cells were susceptible to GSIV and GNNV infection (Wen et al. 2008).

In addition to testing the virus susceptibility on cell lines, several bacterial toxins have also been tested on different cell lines. All three cell lines RGB, RGG and RGH were found sensitive to the extra cellular products of *Photobacterium damselae* ssp. *piscicida* (Ku et al. 2009). The bacterial extra cellular products from *Aeromonas* sp., or *Vibrio anguillarum* were found to be toxic to this SICH cell line (Ahmed et al. 2009a). The RE and CB cell lines were not susceptible to four marine fish viruses. Extra cellular products from *Aeromonas* sp. were toxic to the cell lines (Ahmed et al. 2009b).

Repository of cell lines

To date, out of over 3,400 cell lines deposited at the American Type Culture Collection (ATCC), only 43 cell lines could be found that are of aquatic animals, and only 17 fish cell lines are usable and available for dissemination to the researchers globally. The European Collection of Cell Cultures (ECACC) currently holds over 40,000 cell lines representing 45 different species and 50 tissue types. But only 21 fish cell lines have been listed here. The reluctance to use cell lines stems from researcher's misconception that cell lines are mostly derived from transformed cells and that differentiated characteristics of the tissues of origin are not maintained (Sato 2008). This may be the case for many mammalian cell lines, but most cell lines derived from fish tissues have been from normal tissues with a few exceptions, most notably EPC and RTH-149 cells, which were derived, respectively, from an epithelioma and a hepatoma. Fryer and Lannan (1994) noted that 14 out of 159 fish cell lines reported up to 1994 were initiated from tumorigenic tissues, which is less than 10%. Furthermore, among the fish cell lines listed at ATCC, only three were derived from tumorigenic tissues. This contrasts with mammalian cell lines where over 50% of listed cell lines at the ATCC were derived from cancerous tissues or transformed cells. Altogether ~283 cell lines have been established from finfish around the world but only 43 fish cell lines are being listed in the international cell repository like ATCC, ECACC. If all the established cell lines would have been deposited in that repository, it would be beneficial to the international research community in order to use those cell lines as they are the best alternative to the whole animal research.

Conclusion

Cell cultures, in particular those derived from fish, have been successfully employed as a biological alternative to the use of whole animals. The increasing use and importance of fish cell lines suggest that cell culturists should be encouraged to place these lines with the international cell repositories like ATCC, EACC or other appropriate repository in order to provide a dependable, high-quality source of cells for the benefit of all.

The number of publications containing spurious data as a result of overpassaged, misidentified, or contaminated cell lines is unknown. The basis for any research, development or production program involving cell cultures is the selection of an identity-verified and low-passage cell line. The use of similar and identified passage numbers throughout a project will better ensure reproducible results and comparisons between laboratories. To further ensure the use of authenticated cell lines, full cell line documentation, including the source and passage numbers used during experiments, should be submitted for scientific publications. Cell lines are critical components of experiments and should be considered as standard research reagents and given the same care and quality control measures that surround the use of kits, enzymes and other laboratory products commercially obtained.

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