28 Development of marine fish cell lines and stem cell lines: applications in mariculture and marine biodiversity

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In vitro transformed/continuous marine fish cell lines are important for virology, gene expression studies, cytogenetics, as *in vitro* models in toxicology, transgenics, in many other basic studies and in biodiversity conservation. *In vitro* cell culture systems/cell lines derived from marine fish are necessary for isolation and characterisation of viruses and studies on diversity of viruses in marine environment. The main impetus for the development of many of the continuous fish cell lines was to provide the means for isolating and identifying viruses that are the causative agents of epizootics of commercially important species. Unlike other microorganisms, which can be readily grown in artificial nutrient medium, viruses are obligatory intracellular pathogens and their isolation and propagation are totally dependent on the availability of a live host, such as permissive cell cultures. In addition, most viruses are host-specific and tissue-specific, and they can only be isolated and propagated using cell lines established from tissues of the same/related host species. An appropriate cell line is the most important laboratory tool to grow, isolate, characterise, and identify pathogenic fish viruses. With few exceptions, fish viruses can be replicated only in fish cell cultures.

Cell cultures may be derived from primary explants or dispersed cell suspensions. Because cell proliferation is often found in such cultures, the propagation of cell lines becomes feasible. A monolayer or cell suspension with a significant growth fraction may be dispersed by enzymatic treatment or simple dilution and reseeded, or subcultured, into fresh vessels. This constitutes a passage, and the daughter cultures so formed are the beginning of a cell line. The formation of a cell line from a primary culture implies (1) an increase in the total number of cells over several generations and (2) the ultimate predominance of cells or cell lineages with the capacity for high growth, resulting in (3) a degree of uniformity in the cell population. The line may be characterized, and the characteristics will apply for most of its finite life span. The derivation of continuous (or ''established,'' as they were once known) cell lines usually implies a phenotypic change, or transformation. The alteration in a culture that gives rise to a continuous cell line is commonly called *In vitro transformation* and may occur spontaneously or be chemically or virally induced. Continuous cell lines are usually *aneuploid* and often have a chromosome number between the diploid and tetraploid values. There is also considerable variation in chromosome number and constitution among cells in the population *(heteroploidy)*. It is not clear whether the cells that give rise to continuous lines are present at explantation in very small numbers or arise later as a result of the transformation of one or more cells.

Cell lines or cell strains may be propagated as an adherent monolayer or in suspension. Monolayer culture signifies that, given the opportunity, the cells will attach to the substrate and that normally the cells will be propagated in this mode. Monolayer culture is the mode of culture common to most normal cells, with the exception of hematopoietic cells. *Anchorage dependence* (i.e., attachment to and usually, some degree of spreading onto the substrate) is a prerequisite for cell proliferation in monolayer culture. Suspension cultures are derived from cells that can survive and proliferate without attachment *(anchorage independent)*; this ability is restricted to hematopoietic cells, transformed cell lines, and cells from malignant tumors. It can be shown, however, that a small proportion of cells that are capable of proliferation in suspension exists in many normal tissues. The identity of these cells remains unclear, but a relationship to the stem cell or uncommitted precursor cell compartment has been postulated. This concept implies that some cultured cells represent precursor pools within the tissue of origin. Cultured cell lines are more representative of precursor cell compartments *in vivo* than of fully differentiated cells, as, normally, most differentiated cells do not divide.

Most of the established fish cell lines were derived from cold water fish, such as salmonids, channel cat fish and

common carp. However, many new continuous cell cultures are constantly being developed as a result of intensive efforts in several parts of the world, to provide cell cultures from local species utilised in aquaculture. The present knowledge on various aspects of fish viral diseases has come mainly from temperate countries from fishes like salmon, trout and channel cat fish. The rapid expansion of aquaculture and associated viral diseases in North America, Europe and Japan led to the subsequent development of several fish cell lines for health management purposes. RTG-2 cell line of rainbow trout, *Salmo gairdneri*, gonad origin initiated in 1960 was the first permanent fish cell line to be developed (Wolf and Quimby, 1962). Clem *et al*. (1961) initiated trypsinised blue-striped grunt *Haemulon flavolineatum* fin cultures which provided GF-1 cells, the first line of marine fish origin. Several cell cultures and cell lines from a variety of fishes have been developed since the first cell line from rainbow trout. Teleost cells are the 2nd most numerous among the animal cell lines which have been developed, mammalian cells being the most numerous. A comprehensive list of most fish cell lines developed before 1980 has been published (Wolf and Ahne, 1982). In addition, several comprehensive reviews of maintenance and application of cell cultures all from temperate fishes are available.

Examples of widely used fish cell cultures

Cell line	Fish and tissue of origin
RTG - 2	Rainbow trout gonad
CHSE-214	Chinook salmon embryo
BF-2	Bluegill fry - caudal peduncle
FHM	Flathead minnow - caudal peduncle
BB	Brown bullhaead - caudal peduncle
CAR	Gold fish fin
EPC	Common carp - Epithelioma papulosum cyprini
E11	Snakehead fin
SSN-1	Snakehead fin
SBK-2	Seabass kidney

Although a large number of fish cell lines have been established for isolating and identifying fish viruses (Fryer and Lannan, 1994), relatively few marine fish cell lines are available (Chi *et al*., 1999). Recent interest in marine fish cytogenetics, immunology and pathology, together with other *in vitro* applications, has given rise to the need for improved methods for the isolation, handling and culture of cells from marine fish. The limited numbers of reports on viruses from marine fish compared with those from freshwater fish are due to the shortage of fish cell lines derived from marine fish. The research on marine fish cell lines has progressed rapidly in recent years and several cell lines from tissues of commercially important marine fish have been described.

Three continuous cell lines have been established from gonads of Japanese striped knife jaw, *Oplegnathus fasciatus* (JSKG cell line), embryos of hybrid of kelp *(Epinephelus moara)* and red spotted grouper *E. akaara* (KRE cell line) and skin of greater amberjack *Seriola dumerili* (PAS cell line) (Fernandez *et al*., 1993). A continuous cell line (SAF-1) was developed from fin tissues of an adult gilt head sea bream, *Sparus aurata* (Bejar *et al*., 1997). The GF-1 cell line derived from the fin tissue of the grouper, *Epinephelus coioides* (Hamilton) (Chi *et al*., 1999) can effectively proliferate fish nodavirus and is a promising tool for studying fish nodavirus. Two iridovirus-susceptible cell lines were established and characterised from kidney and liver tissues of the grouper, *Epinephelus awoara*. These cell lines have been designated GK and GL, respectively (Lai *et al*., 2000). A tropical marine fish cell line (SF) was established from fry of Asian seabass, *Lates calcarifer* (Chang *et al*., 2001).

Kang *et al*. (2003) established and characterised two cell lines, FFN cells from the fin tissue and FSP from the spleen tissue of the flounder, *Paralychthys olivaceus*. Both the cell lines were found susceptible to a wide range of fish viruses such as IPNV, marine birna virus, chum salmon virus, IHNV, SVCV and hirame rhabdovirus. Four tropical marine fish cell lines were established from the eye, fin, heart, and swim bladder of the grouper, *Epinephelus awoara* by Lai *et al*. (2003). A continuous cell line, TEC (turbot embryonic cell line), was established from embryos at the gastrula stage of a cultured marine fish, turbot *(Scophthalmus maximus)* (Chen *et al*., 2005). Qin *et al*. (2006) described the development and characterisation of a tropical marine fish cell line (GS), derived from the spleen of orange spotted grouper, *Epinephelus coioides*. It is suggested that the GS cell line has good potential as a diagnostic tool for isolation and propagation of iridovirus and nodavirus. When the GS cells were transfected with pEGFP vector DNA, significant fluorescent signals were observed suggesting that the GS cell line can be used as a useful tool for transgenic and genetic manipulation studies.

Fish tissue culture work is relatively new in India. Sathe *et al*. (1995) established a cell line (MG-3) from gills of mrigal, *Cirrhinus mrigala* and characterised it with respect to isoenzyme pattern and chromosome number. Moreover, electron microscopic studies were also carried out which revealed the cellular structure and also secretory nature of the cultured cells. Sathe *et al*. (1997) have also developed a cell line from gill of rohu, *Labeo rohita*. Primary cultures were developed from the kidney of freshwater fish, *Heteropneustes fossilis*, by employing certain modifications in conventional procedures and a number of clones of cells have been developed (Singh *et al*., 1995). Lakra and Bhonde (1996) were successful in developing primary cultures from the caudal fin of rohu, *Labeo rohita*. Primary cultures from various tissues of

Indian major carps (Rao *et al*., 1997), caudal fin of *Tor putitora* (Prasanna *et al*., 2000), and ovary of *Clarias gariepinus* (Kumar *et al*., 2001) have also been reported. Unfortunately, none of these cell lines/cell cultures were maintained for long-term applications (Kumar *et al*., 2001). Kumar *et al*. (2001) developed a cell culture system from the ovarian tissue of African catfish, *Clarias gariepinus*. The cell culture could be passaged 15 times after which they ceased to multiply and consequently perished.

Lakra *et al*. (2005) assessed the potential of six tissues of *Labeo rohita* viz. kidney, liver, heart, gill, caudal fin and swim bladder for attachment, cell growth and proliferation by explant culture and trypsinisation methods. Lakra *et al*. (2006) reported the development of a diploid cell line (TP-1) for the first time from fry of golden mahseer, *Tor putitora*. Sahul Hameed *et al*. (2006) established and characterised India's first marine fish cell line (SISK) from kidney of sea bass, *Lates calcarifer*. The cell line was found to be susceptible to two marine fish viruses. Parameswaran *et al*. (2006), described the establishment of an embryonic cell line from Asian sea bass (SISE) derived from blastula-stage embryos of *L. calcarifer.* Parameswaran *et al*. (2006) also described the development and characterisation of a marine fish cell line (SISS), derived from the spleen of sea bass. Two cell culture systems namely epithelioid cells of *Lates* (LCE) and fibroblastic cells of *Lates* (LCF) have been developed from fry and fingerlings of *L. calcarifer* (Lakra *et al*., 2006).

Fish embryonic stem cell lines

Embryonic stem (ES) cells are undifferentiated cells derived from early developing embryos of animals, characterised by their capacity for self renewal and pluripotency. These cells retain their pluripotency after long-term cultivation *in vitro* and can be induced to differentiate into a variety of cell types. When introduced into host embryo, the ES cells can participate in normal development and contribute to several tissues of the host, including cells of the germ line. These characteristics make ES cells ideal experimental systems for *in vitro* studies of embryonic cell development and differentiation and as vector for the efficient transfer of foreign DNA into the germ line of an organism. In addition, ES cells provide an attractive strategy for the preservation of biodiversity (Hong *et al*., 1996). These cells have the potential to produce any type of cell of the body and can be propagated in unlimited quantities, which led to the importance of human ES cells (hESCs) in regenerative medicine and treatment of a variety of diseases.

ES cells provide unique tool for cell-mediated gene transfer and targeted gene mutations due to the possibility of *In vitro* selection of desired genotypes. Though the ES cell approach has up to now been limited to mice, there is an increasing interest to develop this technology in both model and commercial fish species, with so far promising results in the medaka *(Oryzias latipes)* and zebrafish *(Danio rerio)*. Pluripotent embryonic stem cells (ES) provide an efficient approach for genome manipulation with many applications in marine biotechnology and developmental studies. The methods of derivation, characterization and behaviour of fish ES cells in *In vitro* culture are known only for a very limited species of fish world over. Chen *et al*. (2003) developed a pluripotent cell line, LJES1 from blastula stage embryos of the sea perch, *Lateolabrax japonicus*. Other than the report on attempts for derivation of ES cells from *Lates calcarifer*, *Labeo rohita* and *Catla catla* (Parameswaran *et al*., 2007; Dash *et al*., 2008; 2010) there is no published report available on development of ES cell lines from marine fish in India.

Cryo-preservation of fish cell lines

Cell culture systems are biological entities with specific physiological needs, much like any other laboratory animals. They require ongoing care, adequate nutrition, a proper environment and regular check ups. Every cell line cultured must also be backed up by cells in frozen storage. Therefore cryopreservation of fish cell lines is very important. Optimal freezing of cells for maximum viable recovery on thawing depends on minimizing intracellular ice crystal formation and reducing cryogenic damage from foci of high concentration solutes formed when intracellular water freezes. This is achieved by (1) slow freezing, (ii) by using a hydrophilic cryoprotectant to sequester water, (iii) by storing the cells at the lowest possible temperature and (iv) by thawing rapidly to minimize ice crystal growth and generation of solute gradients formed as the residual intracellular ice melts. Freezing in liquid nitrogen or in ultra cold freezers is the method of choice for storage of fish cell lines. The cell suspension is frozen in the presence of a cryoprotectant such as glycerol or dimethyl sulfoxide (DMSO). Of these two, DMSO appears to be the more effective, possibly because it penetrates the cell better than glycerol. Concentrations between 5% and 15% have been used, but 7.5% or 10% is more ideal. The medium used for freezing fish cell lines generally contains 10% or more serum and either of the two cryoprotectants - glycerol or dimethyl sulphoxide (DMSO) added to a final concentration of around 10%.

Suggested reading

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