APPLIED ASPECTS OF FISH PHYSIOLOGY

Cellular, Molecular, Genomics, and Biomedical Approaches

Contents

Culture of Fish Cell Lines

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Glossary

Basal media A solution of buffering agents and of nutrients, which include a hexose, bulk ions, trace elements, amino acids, and vitamins.

Cell line Begins with the successful passaging of a primary culture.

CO_2 incubator A chamber that maintains temperatures a few degrees above ambient to approximately 40 °C and allows the injection of one or more gases, usually CO_2.

Cryopreservation Storing cells, usually in 10% dimethyl sulfoxide, at temperatures below −80 °C.

Culture vessels Commonly plastic flasks and also glass or plastic petri dishes.

Ecotoxicology An interdisciplinary science dealing with toxicants in the environment.

Epithelial-like cell line Majority of the cells attach and spread on the growth surface to have shapes like irregular patio blocks.

Fibroblast-like cell line Majority of the cells attach and spread on the growth surface to have long bipolar shapes.

Finite cell line Cells can undergo a limited number of population doublings.

Heat inactivation of serum Incubation at 56 °C for 30 min.

Immortal cell line Cells can be propagated indefinitely and are as same as a continuous cell line.

Laminar flow hood A cabinet in which sterile air is created by passing the air through a high-efficiency particle air filter in smooth or laminar flow and blown across a work area to create a sterile environment in which cultures can be manipulated.

Line Same as cell line.

Macrophage-like cell line Majority of the cells express some macrophage properties, such as being highly phagocytic and having major histocompatibility class II proteins on the cell surface; often, proliferate loosely attached to the growth surface.
Passaging Transfer of cells from one culture vessel to another. This is synonymous with subculturing, subcultivating, or splitting.
Primary cultures A culture initiated directly from the cells, tissues, or organs of an animal.
Proliferation An increase in cell number through cell division.
Senescence Loss by cell lines of the ability to be propagated.
Serum The fluid fraction after blood has been allowed to coagulate and trap blood cells with clotting factors into a solid clot.
Spheroid An aggregate of cells in suspension within a culture.
Spontaneous immortalization Cells acquire the ability to be grown indefinitely through only the routine maintenance of the cells in culture.
Trypsin A protease from the pancreas that is used to passage cells.
Xenobiotic metabolism Changes to foreign compounds brought about by cells or organisms.

Introduction

Cell lines are cultures of animal cells that can be propagated repeatedly and sometimes indefinitely. They arise from primary cell cultures. Primary cultures are initiated directly from the cells, tissues, or organs of animals and are typically used in experiments within a few days. By convention, the passaging or subcultivation of a primary culture begins a cell line. However, not all primary cultures yield cell lines. Instead, the cells of some subcultivated cultures die off slowly. Why the cells of other cultures keep on growing and allow these cultures to be continuously subcultivated is a complex and incompletely understood subject. The subject is intensively studied with mammalian cells because of the relevance of this to the mechanisms of cellular senescence and the origins of cancer cells. In the case of mammalian cell lines, some are finite. They can only be subcultivated a limited number of times before they senesce. Yet other mammalian cell lines are continuous or immortal and can be grown indefinitely. Continuing with the case of mammals, some primary cultures or finite cell lines can be experimentally immortalized to become continuous cell lines.

By contrast, with fish, little is known about cellular senescence and immortalization. Fortunately and surprisingly, many fish cell cultures have been subcultivated indefinitely. These cell lines are said to have become spontaneously immortalized. This is how nearly all fish cell lines have arisen. Most of these have been derived from bony fish, but they are called simply fish or piscine cell lines. The development of fish cell lines is described elsewhere (see the 'Further reading' section). Here, the sources and types of fish cell lines are noted and what is needed to maintain these cell lines is described, ending with a brief overview of how the cell lines are being used.

Sources

Like mammalian lines, fish cell lines can be stored frozen, aiding their distribution to researchers around the world. How piscine cell lines are cryopreserved is discussed later in the 'Cryopreservation' section. A recent list of repositories with fish cell lines has been presented (see the 'Further reading' section). Relative to the demand for human and rodent cell lines, the request for piscine cell lines is limited and so the number of fish lines in these cell banks is small. Despite the restricted choice, repositories are the best cell line sources. They assure quality and reliable delivery, but of course at a price.

Cell lines have been developed from most but not all tissues and organs of bony fish, with only a few from tumors, usually liver tumors. Most piscine cell lines are yet to be analyzed for differentiated properties and are described simply as being either epithelial-like or fibroblast-like. Exceptions are some lymphocyte and macrophage cell lines. Figure 1 illustrates the morphologies in cultures of salmonid epithelial-, fibroblast-, and macrophage-like cell lines. Other properties commonly examined are the optimal growth conditions and the karyotype. Although diploid fish

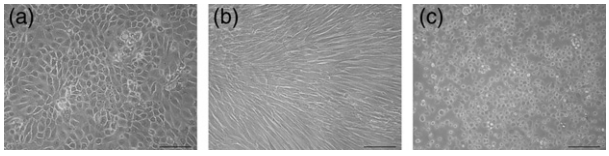

Figure 1 Phase-contrast microscopy appearance of cultures of an epithelial-like (a), a fibroblast-like (b), and a macrophage-like (c) cell line. The lines are RTgutGC from the epithelial layer of the rainbow trout intestine (a), AS20imf from the subepithelial layer of the Atlantic salmon intestine (b), and RTS11 from the rainbow trout spleen (c). Scale = 100 μm.

cell lines do exist, most cultures are heteroploid: they contain cells with chromosome numbers that are not an exact multiple of the haploid number.

Approximately 10 times as many fish cell lines have been described in the literature as have been placed in repositories. Lists of these have been prepared at several points over the last 30 years. Whereas ~29 000 species of bony fish in over 500 families have been classified, cell lines have been prepared from only about 75 species in 35 families. In the past, methods of confirming that a cell line was indeed from the stated starting species were problematic, but now species confirmation can be done reliably by DNA barcoding. This has revealed at least one surprise: epithelioma papulosum cyprini (EPC), which was thought to be a carp (*Cyprinus carpio*) cell line, is fathead minnow (*Pimephales promelas*). Over 10 different cell lines have been developed from each of several species, including catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), and zebrafish (*Danio rerio*). The total number of fish cell lines in the literature is now reaching close to 200, but many of these have likely been lost. Yet others are still available from the community of scientists developing and using piscine cell lines.

Whether from a repository or a colleague, the lines can be obtained as either a vial of frozen cells or as a flask of living cells. Sending flasks of cells is often easier because vials need to be embedded in dry ice, which imposes shipping restrictions and increases the shipping cost. Sending flasks of cell lines from coldwater fish in the summer might require placing the flask on an ice pack, but this poses no shipping problems. Getting living cultures is also easier. The cultures can often be subcultivated shortly after being received, and the problem of thawing the cells, the success of which can be variable, is avoided.

Equipment

At least three pieces of equipment are required to routinely maintain piscine cell lines. An inverted phase microscope is needed in order to visually inspect cultures. For passaging, a sterile environment is needed in order to open culture vessels and to remove or add cells without contaminating cultures with microbes. This is achieved in the working area of a laminar flow hood, which can be either vertical or horizontal, with the latter being less expensive. A vertical hood provides a current of sterile air between the worker and working area, protecting the worker from being exposed to any human pathogen that might inadvertently be associated with the cells. As there is no record of fish cell cultures containing human pathogens, a horizontal hood is often used. The third piece of equipment is a low-speed centrifuge to collect cells either for routine subcultivation or to set up experimental cultures. As discussed below, an incubator may or may not be required.

Culture Vessels

A vast array of plastic tissue culture ware has been developed for mammalian cells and nearly all these items work well with fish cell lines. Some of these items, such as flasks and petri dishes, are used for routinely growing cells, whereas others are used to monitor a particular endpoint. For the routine growth of piscine cell lines, nonvented flasks are favored because the loss of medium through evaporation is less. For RTS11, the most consistent growth is achieved with 12.5 cm^2 flasks, whereas for most other salmonid cell lines, 75 cm^2 flasks are convenient. In these, the cells grow to completely cover the flask surface and from a single flask, several multiwell plates can be set up for experimental purposes. With flasks >75 cm^2, RTL-W1 and RTgill-W1, and rainbow trout cell lines, are slow to cover the growth surface and often leave unfilled patches in the center.

Most plastic vessels are treated to promote animal cell attachment and proliferation, but some have hydrophobic surfaces to keep the cells in suspension. All these work for fish cell lines. In vessels with hydrophobic surfaces, suspended epithelial- and fibroblast-like cell lines aggregate over a few hours to form spheroids (**Figure 2**). Fish cells also attach to glass petri dishes. However, salmonid cell lines have been found to spread poorly in Lab Tek Chamber Slides (Nunc) with a glass surface, but do spread well when these slides have a plastic Permanox surface.

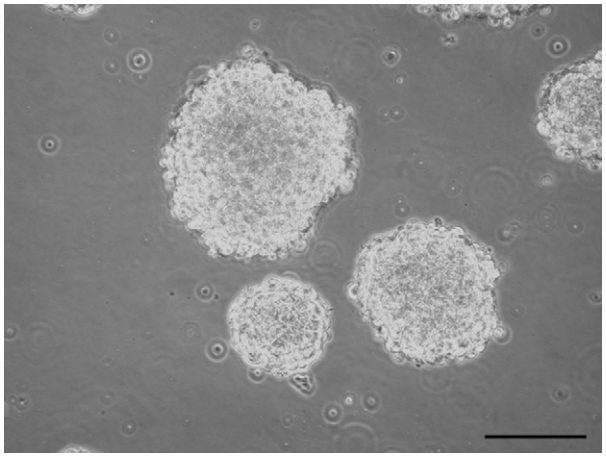

Figure 2 Phase-contrast microscopy appearance of spheroids formed in Nunc low-bind tissue culture dishes (coating of phospholipid polymer, 2-methacryloxyloxyethyl phosphorylchloline). The spheroids were initiated by adding 2×10^6 cells of the rainbow trout spleen stromal cell line (RTS34st) into 60 mm low-bind dishes. The medium was L-15 with 10% FBS, and after 24 h at room temperature, the culture was photographed. Scale = 100 μm.

Growth Media

The complete growth medium for the routine propagation of piscine cell lines has two essential parts, a basal medium and a supplement, with antibiotics being optional.

The basal medium constitutes an aqueous solution of buffering agents and of nutrients, which include a hexose, bulk ions, trace elements, amino acids, and vitamins. Basal media can be purchased, already made sterile in tissue-culture-grade water, or with all the constituents packaged as powders that need only be mixed with water. Some basal media are sold without glutamine because of its instability, and these are completed just before use by adding aliquots of a sterile concentrated glutamine solution (200 mM). Many different basal media are available commercially, and all were originally developed and optimized for mammalian cells. Fortunately, cells from other vertebrates, including both freshwater and marine fish, do well in them.

The choice of basal media for fish cells usually revolves around the availability of a CO_2 incubator, which is necessary to support the most common type of buffering system in basal media, sodium bicarbonate ($NaHCO_3$). For $NaHCO_3$ to act as buffer, a CO_2-rich atmosphere, usually 5% CO_2 and 95% air, is required and is provided for by a CO_2 incubator. Eagle's minimum essential medium (MEM) in Earle's salts is a basal medium that requires a CO_2 atmosphere and has been used frequently in the past to successfully grow a variety of fish cell lines. However, the use of CO_2 incubators for temperatures below room temperature is problematic for several reasons. As purchased, a CO_2 incubator can only raise the temperature, so a means of cooling has to be found for lower temperatures. The solubility of CO_2 increases at low temperatures, causing media to become acidic.

Two groups of basal media maintain their buffering capacity under normal atmospheric conditions (~0.03% CO_2) and are used to grow piscine cell lines. One group was developed for CO_2 incubators with slight modification. For example, MEM is made up in Hank's salts, which maintains the pH under atmospheric conditions. Another modification is the addition of organic buffers, such as HEPES. Many commercial basal media include either 15 or 25 mM HEPES plus the original concentration of sodium bicarbonate. Although buffering is usually optimal in a 5% CO_2 atmosphere, fish cells grow under atmospheric conditions. Another basal media group was formulated specifically for use in free gas exchange with air. The oldest is Leibovitz's L-15, which maintains physiological pH through a combination of salts, high basic amino acid concentrations, and galactose in place of glucose. A more recent addition is CO_2-independent medium (Gibco or Invitrogen). Unlike L-15, it contains sodium bicarbonate. Most fish cell lines are grown in L-15, but a few fish cell lines maintained in CO_2-independent medium grew well. These basal media are used without a CO_2 incubator.

The most common supplement is serum. Sterile sera from a few farm animals and sometimes fish sera are available commercially. Serum contains hundreds of proteins and is incompletely characterized, so it is described as an undefined supplement. No defined media have been reported yet that consistently support the proliferation of a piscine cell line through many passages.

Serum has to be added to basal media to improve fish-cell proliferation. For example, Atlantic salmon (*Salmo salar*) cell numbers slowly decline in L-15 over days to weeks (**Figure 3**). Yet, when L-15 is supplemented with 10% fetal bovine serum (FBS), cell number increases approximately eightfold over 2 weeks (**Figure 3**). Indeed, FBS with or without heat inactivation is the most common serum used for routine fish-cell growth. The usual FBS concentration is 10%, but ranges from 5% to 30% depending on the cell line. Sera from calves and horses are less expensive and infrequently used because of slower growth. Surprisingly, fish sera are rarely used, perhaps because FBS is cheaper and fish sera were found to be sometimes cytotoxic to fish cells.

For the routine maintenance of cell lines, antibiotics are added to media. Usually, these are penicillin at 100 IU with streptomycin at 100 ug ml^{-1} (penicillin/streptomycin). An alternative is to use gentamycin at 50 ug ml^{-1}. Less frequently, an antifungal agent, amphotericin (fungizone), is added at variable concentrations. The use of antibiotics is often discouraged because consistent antibiotic use can lead to the development of resistant microbes and hide poor aseptic techniques.

Growth Temperature

The temperature range over which fish cells divide in culture is referred to as the proliferation zone and is wide. They tolerate an even wider temperature range,

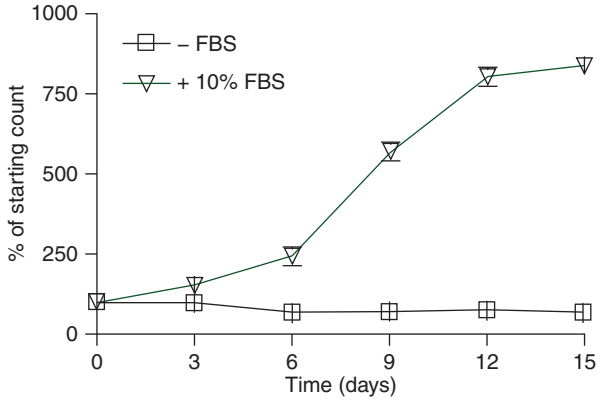

Figure 3 Change in cell number over time at room temperature for cultures of AS20imf in Leibovitz's L-15 either without a supplement (a) or with a supplement of 10% FBS (b). For each time point, cell number was counted with a Coulter counter in three replicate wells of a 12-well plate. The results are expressed as a percentage of the starting cell number, which was ~5.0×10^4 cells.

which has been defined as the endurance zone. The temperature chosen for a cell line depends on the purpose of the cultures, the species, and the availability of incubators. To maximize cell production, an optimal growth temperature should be selected. For cold-water fish, like salmon, this is in the 20–23 °C range; and for warm-water fish, like zebrafish, this is 26–30 °C. If the purpose is to just maintain the cultures for later use, lower temperatures allow the cells to slowly proliferate, reducing culture maintenance.

Nearly all fish cell lines grow at room temperature, which alleviates the need for an incubator or controlled rooms at other temperatures. The incubator type depends on the basal media. With L-15 or CO_2-independent media, a regular incubator can be used. However, if the temperatures are high and the incubator is not humidified, medium can rapidly evaporate from petri dish cultures, increasing the osmolarity and slowing cell growth. Therefore, in this situation and also for room temperature without an incubator, unvented flasks with the caps tightly closed are recommended. When buffering of the basal media is done only with sodium bicarbonate, a CO_2 incubator must be used.

Passaging

Passaging, which is also referred to as subculturing, subcultivating, or splitting, is the transfer of cells from one culture vessel in which the cells have been growing for days or weeks to two or more new culture vessels in which they will again be grown for days or weeks. This is routine maintenance and expands cell number. It is usually done in flasks.

Some fish white-blood cell lines, such as RTS11, grow in suspension or loosely attached to the growth surface and are the easiest to passage. A sterile cell scraper gently dislodges attached cells into the medium with the cells already in suspension. A pipette then removes aliquots of cell suspension into fresh medium.

In contrast to the lymphoid cell lines, most piscine cell lines are anchorage dependent and cells are removed from the flask growth surface before passage with or without enzymes as per commercially available step-by-step protocols. With fish cell lines, these steps are done at room temperature or lower. Trypsin is the most common enzymatic method, but the monolayer of cells must be rinsed with ethylenediaminetetraacetic acid to remove divalent cations prior to 0.01% trypsin being added. Trypsin usually causes the cells to round up and start detaching within 5 min, at which point medium with serum is added to stop the trypsin. Sometimes with older cultures, cells take longer to detach and sometimes cells clump on the addition of serum. Alternative methods include use of TryplE Select or Express (Invitrogen), trypsin-like enzymes produced by microbes. These are more convenient to use than trypsin and among their advantages is that serum need not be added to stop their actions. Nonenzymatic methods of splitting cultures act by chelating divalent cations. A commercial solution for this is cell dissociation solution nonenzymatic (Sigma), which is used to successfully passage several fish cell lines. Regardless of the dissociation method, a split ratio of one old confluent flask to two new flasks is common for slow-growing adherent cell lines from cold-water fish. With salmonid cell lines at room temperature, cultures can be commonly split one to two every 7–10 days. Higher split ratios can be used for more vigorously growing cell lines, such as EPC.

Cryopreservation

Cryopreservation of cell lines provides a supply of cells for some later date and involves the same methods used for mammalian cell lines. The usual cryoprotectants are dimethyl sulfoxide and glycerol at 5–10% (v/v) in the complete growth medium. Indefinite storage is done in Dewars of liquid N_2 at −196 °C, but cells store for approximately a year in electrical freezers at −80 °C. Cells are usually frozen slowly in several ways. One uses programmable coolers set at a cooling rate of often −1 °C min^{-1}, but these are not usually available. Alternatively, vials in a polystyrene box, to ensure slow freezing, are placed in a −70 to −90 °C freezer for at least 2 h before being transferred to liquid N_2. A third strategy is to place vials in the atmosphere above the liquid N_2 in the Dewar and then after 2–24 h lower them into the liquid N_2. Rapid thawing and speedy cryoprotectant removal are key to getting high proportions (70–90%) of living cells, although even low viability (~10%) can lead to proliferating cultures of some fish cell lines.

Applications

The uses to which fish cell lines are being put keeps increasing. The first and most common application is to study viral diseases. The fastest growing area of use is in toxicology and ecotoxicology. Other research disciplines making use of fish cell lines include biomedicine, immunology, and animal physiology. Some applications defy easy classification, such as the use of a goldfish fibroblast cell line to explore the production of fish filets for long voyages in space.

See also: **Aquaculture**: Physiology of Fish in Culture Environments. **Toxicology**: The Toxicology of Organics in Fishes.

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