Brief Communication

Establishment of immortal marine fish cell lines as *in vitro* tools for advancing research in environmental monitoring, biotechnology and biodiversity conservation

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Immortalized cell lines are invaluable to researchers as they can proliferate indefinitely, allowing them to be cultured across multiple generations. Unlike primary cells with a limited lifespan, immortal cell lines (also called as continuous cell lines) bypass challenges such as ethical concerns, difficulty in extraction, limited passage ability, and inconsistent results due to variations in cell sources. Most cells in laboratory conditions face the Hayflick limit, where telomeres shorten with each division, leading to senescence. Immortalized cell lines overcome these limitations, enabling stable, long-term studies while eliminating the need for repeated cell isolation and cultivation, thus saving both time and resources in research. Continuous cell lines can proliferate indefinitely *in vitro*, providing a sustainable and reproducible system for a wide range of research purposes. These cell lines offer a unique platform for investigating organisms at the cellular and molecular levels, providing insights that are not always possible with whole-organism models. It replicates the cellular and genetic homogeneity of the host, while minimising the variability inherent with *in vivo* systems. Consequently, there has been a growing focus on developing new cell lines to support a broader array of biological investigations.

The development of animal cell cultures was initially driven by the need for antiviral vaccine production and cancer research (Freshney, 2010). Similarly, fish cell cultures were initially established to support the growth of viruses for studying fish diseases. Fish cells possess unique traits such as tolerance to low oxygen, wide temperature adaptability, strong intracellular buffering, and high lactate dehydrogenase (LDH) activity, making them ideal for *in vitro* studies. The first permanent fish cell line, RTG-2, derived from rainbow trout gonadal tissue in 1960 (Wolf and Quimby, 1962), paved the way for development of a diverse range of species and tissue-specific fish cell lines. These have become invaluable tools in toxicology, immunology, virology, and genetics, facilitating research into cellular responses to environmental stress, pathogens, and pollutants, as well as for studying gene expression, protein synthesis, and metabolism.

The marine environment hosts a vast diversity of fish species that play vital roles in global biodiversity, fisheries, and mariculture. As research on marine fish species expands, there is a rising interest in developing *in vitro* tools, including marine fish cell lines, to explore their biology and responses to environmental shifts. For marine species that are difficult to access or maintain in the lab, cell lines provide a practical alternative to whole animal studies, enabling detailed investigations into how marine fish cells respond to stressors like climate change, pollution, and disease outbreaks.

Development of marine fish cell lines

The development of fish cell lines involves isolating cells from fish tissues, adapting them to artificial culture

environments, as well as maintaining and passaging them over extended periods. Establishing a stable cell line requires careful optimization of both culture media and growth conditions. Typically, cell lines originate from primary cultures, which are derived directly from cells, tissues, or organs of an organism. Primary cultures of fish cell and tissues can be initiated by one of the two widely used methods viz., plating multiple explants (explantation) and trypsinisation (enzymatic digestion, using enzymes such as trypsin). Cell populations of primary cultures are usually heterogeneous than those of cell lines. At ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), we have successfully developed and characterized 16 continuous cell lines derived from commercially important marine fish species, including ornamental varieties, using either explant or trypsinization methods. These cell lines are derived from a variety of tissues spanning gill, fin, spleen, brain, heart, muscle, liver as well as embryonic tissue of various commercially important marine fish species.

Cell culture media and supplements

In *in vitro* cell cultures, growth media provide essential nutrients for cell proliferation, and a variety of complete media are commercially available. Media formulated for mammalian cells can also support fish cell growth when supplemented with foetal bovine serum (FBS). While antibiotics like penicillin and streptomycin are often added, fish cell lines can be maintained without them. Leibovitz's L-15 medium, which regulates pH through salts and high amino acid concentrations, is widely used for fish cell lines, especially with increased NaCl for marine species (Bols and Lee, 1994). FBS, though the most expensive component, is crucial for cell culture. For marine fish cell culture at ICAR-CMFRI, Leibovitz's L-15 medium supplemented with FBS is routinely used.

Subculture and passage

Monolayers initiated through explantation or enzymatic dissociation are subcultured once they reach confluency. This is done either by enzymatic or mechanical dispersion. We employ enzymatic dispersion, with trypsin-EDTA for subculture. With each subsequent subculture, cells with the fastest proliferation rate gradually dominate, while slower-growing or non-proliferating cells are diluted out. During early passages, 15-20% FBS is used, with 5% being sufficient in later stages.

Spontaneous immortalization of fish cell lines

Cell cultures derived from normal tissues undergo senescence after a set number of divisions due to the progressive shortening of telomeres, which prevents further replication. Exceptions include germ cells, tumour cells, and transformed cell lines, which produce telomerase, an enzyme that maintains telomere length, allowing indefinite division. All our marine fish cell lines are generated through spontaneous immortalization, where cells continued to proliferate without any addition of biological, chemical, or physical agents. Unlike mammalian cells, which show senescence markers like SA β-Gal, fish cells do not develop these markers with continued passaging. Spontaneous transformation of our cell lines generally happens between 50 to 80 passages, which is evidenced by an increase in plating efficiency and reduced FBS requirement. All our immortalised cell lines are adapted to grow in L!5 medium supplemented with 2% FBS.

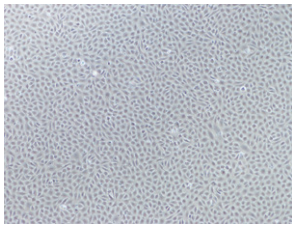
Cryopreservation

The freezing medium for fish cells typically includes 20% or more serum, along with a cryoprotectant, either glycerol or dimethyl sulfoxide (DMSO). We use DMSO at a final concentration of 10% as cryoprotectant and the cells are stored in liquid nitrogen at -196°C, which allows for indefinite preservation. The sixteen marine fish cell lines developed in ICAR-CMFRI have undergone *in vitro* passages ranging from 88 to 471, as outlined in Table 1. All these cell lines are anchorage dependent and grow adherent to the surfaces of tissue culture vessels. The cell lines have been cryopreserved in liquid nitrogen at different passage levels. The cell lines are predominantly fibroblast or epithelial in nature and have been thoroughly characterized. The immortalised cell lines are grown routinely in L-15 medium with 2% FBS at ambient temperature of 28±2°C. All the sixteen marine fish cell lines developed at CMFRI are being used for screening marine fish viruses, conducting gene transfection studies, performing cytotoxicity assessments on environmental contaminants and bacterial toxins, as well as for cell-based seafood research. Out of the 16 cell lines, 12 have been deposited in the National Repository of Fish Cell Lines (NRFC) in Lucknow, India, with their respective accession numbers provided (Table 1).

Table 1. List of Marine fish cell lines developed in ICAR-CMFRI

Cell line code	Species and tissue of origin	Number of passages crossed *in vitro*	NRFC (Lucknow) Accession number	NCBI GenBank Accession number
EM3GEx	*Epinephelus malabaricus,* Gill explant	391	NRFC032	MK165214
EM4SpEx	*Epinephelus malabaricus,* Spleen explant	471	NRFC033	MK165217
EM2HTr	*Epinephelus malabaricus,*Trypsinised Heart	298	NRFC030	MK165216
EM2GEx	*Epinephelus malabaricus,* Gill explant	340	NRFC031	MK165215
DT1CPEx	*Dascyllus trimaculatus,* Caudal peduncle muscle explant	419	NRFC024	KP791798
DT1F4Ex	*Dascyllus trimaculatus,* Fin explant	359	NRFC025	KP791797
DT1CPTr	*Dascyllus trimaculatus,* Trypsinised Caudal peduncle muscle	434	NRFC026	KP791799
PC1CpTr	*Pomacentrus caeruleus* Trypsinised Caudal peduncle muscle	318	NRFC035	KY982626
PC1F1Ex	*Pomacentrus caeruleus,* Fin explant	324	NRFC036	KY982627
PC1L1Tr	*Pomacentrus caeruleus,* Trypsinised Liver	311	NRFC037	KY982628
RC4H1Tr	*Rachycentron canadum,* Trypsinised Heart	290	NRFC027	MH559419
EB2SpEx	*Epinephelus bleekeri,* Spleen explant	333	NRFC038	MK165218
CA1F3Ex	*Cromileptes altivelis,* Fin explant	161	Not deposited	OM131589
CA1F4Tr	*Cromileptes altivelis,* Trypsinised Fin	88	"	OM131590
PB1BrTr	*Premnas biaculeatus,* Trypsinised Brain	108	"	OR290980
AP7EF1	*Amphiprion percula,* Embryo	99	"	OM127852

Phase contrast photomicrographs of marine fish cell lines developed at ICAR-CMFRI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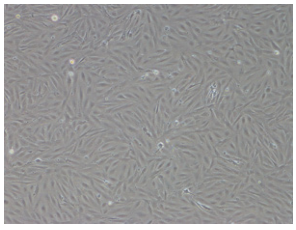
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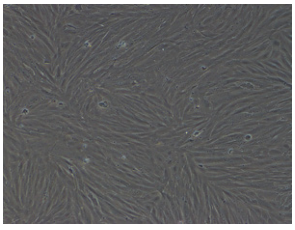
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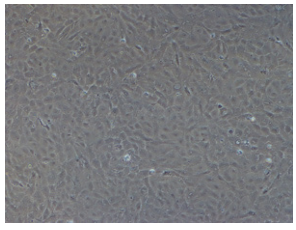
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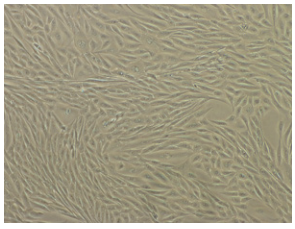
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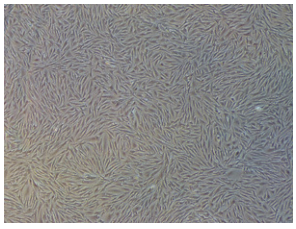
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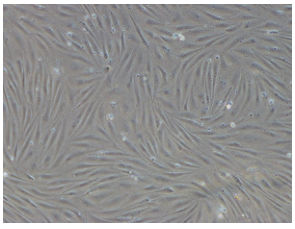
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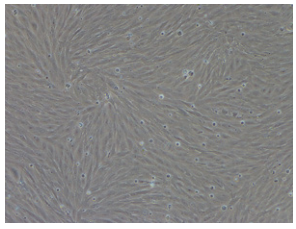
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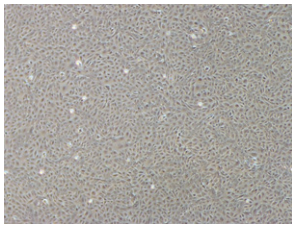
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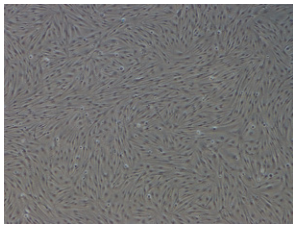
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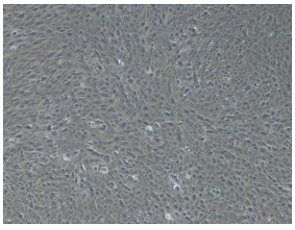
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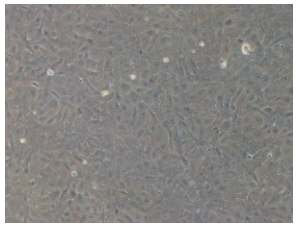
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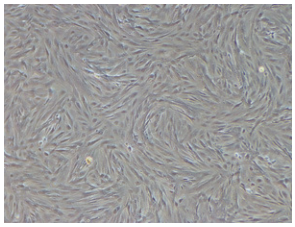

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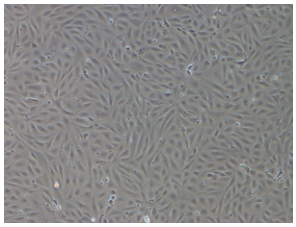

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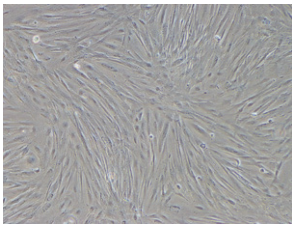

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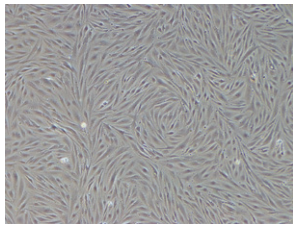


AP7EF1

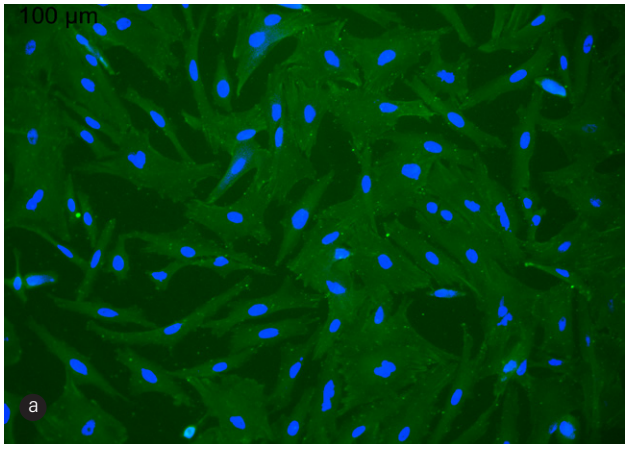
Characterisation of cell lines

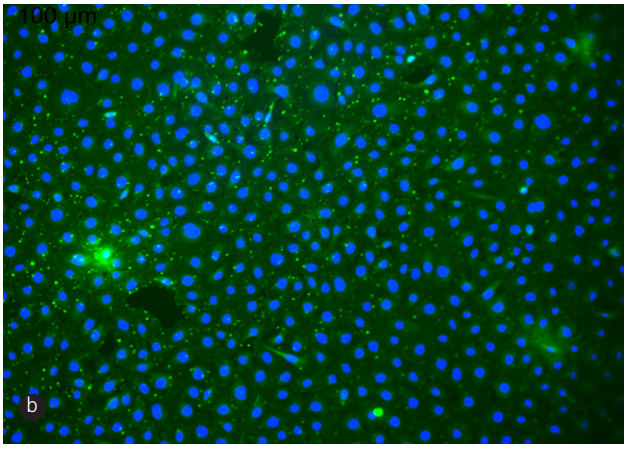
Optimisation of growth conditions

By systematically assessing and optimizing growth conditions, we can better understand the specific requirements of each cell line, ultimately enhancing their performance in experimental settings. All the cell lines developed have been optimised for key parameters such as temperature, and FBS concentration, along with the determination of plating and seeding efficiencies, which are critical for successful cell culture.

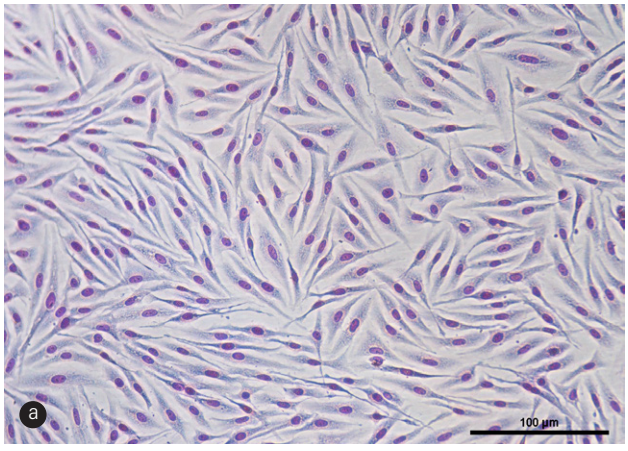
Cell morphology and ultrastructure

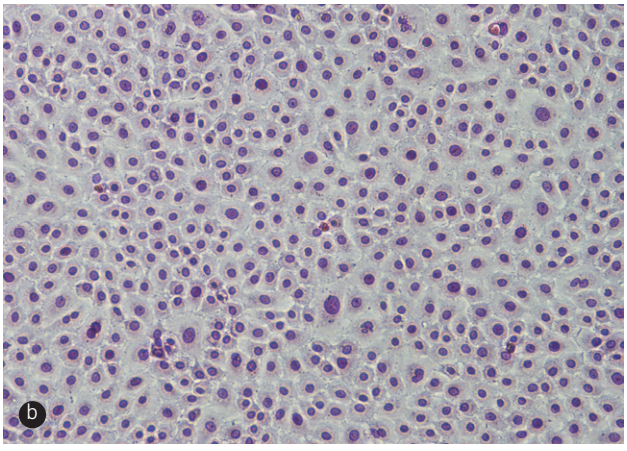
In fish cell cultures, the predominant cell shapes are typically epithelial-like or fibroblast-like, which may indicate their origin from epithelial or connective tissue. Fibroblast-like cells are bipolar, elongated, and often align in parallel as they reach confluency. In contrast, epithelial-like cells are flat with irregular to cobblestone outlines, and their shape can be variable. To correlate the epithelial and fibroblast shapes with cytoskeletal component expression, we have employed immunofluorescence staining using commercial antibodies against cytoskeletal proteins such as Vimentin and Pancytokeratin. Additionally, Giemsa staining and ultrastructural



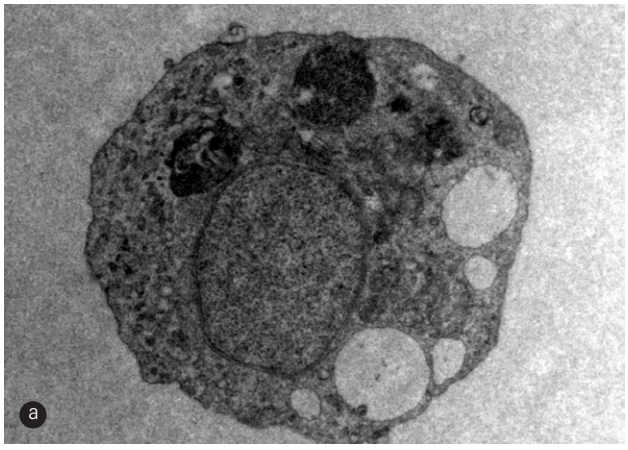


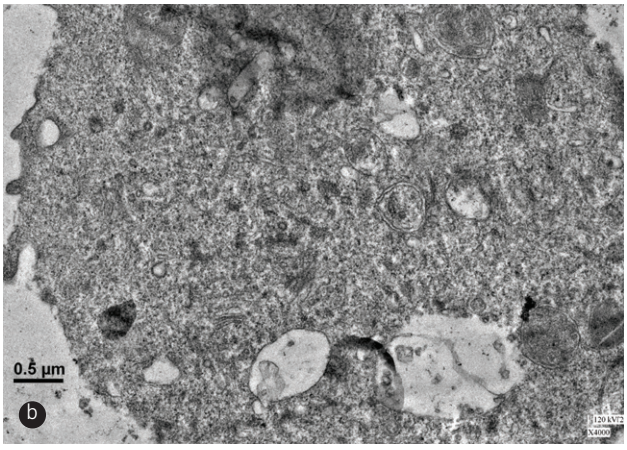
Immunofluorescence staining of (a) Fibroblast and (b) Epithelial markers





Photomicrographs of Giemsa stained (a) Fibroblast and (b) Epithelial cells





TEM images of cells revealing (a) cell structure and (b) organelles

studies *via* transmission electron microscopy (TEM) were also employed to further assess cell morphology and structure.

Cell line authentication and confirmation of species of origin

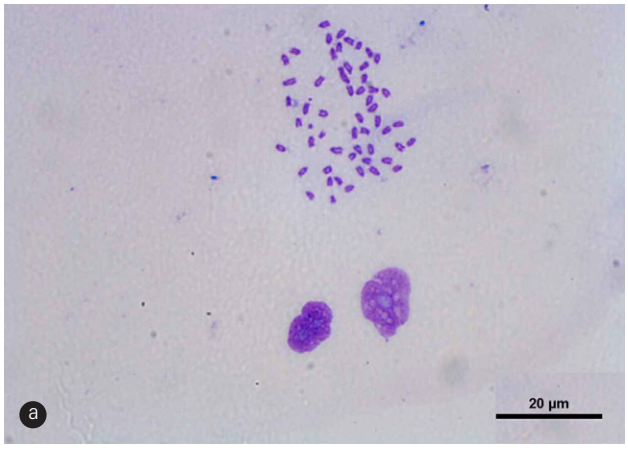
Cell lines are authenticated to confirm their species of origin and ensure the absence of cross-contamination, often using mitochondrial CO1 gene sequence analysis. Amplification and sequencing of a 653 bp region of the mitochondrial CO1 gene is effective for unambiguously identifying fish species (Ward *et al*., 2009). This DNA barcoding technique is widely used to authenticate species identity of fish cell lines. We have confirmed the species of origin for all cell lines through partial sequencing of the mitochondrial CO1 gene, with the sequences deposited in NCBI GenBank.

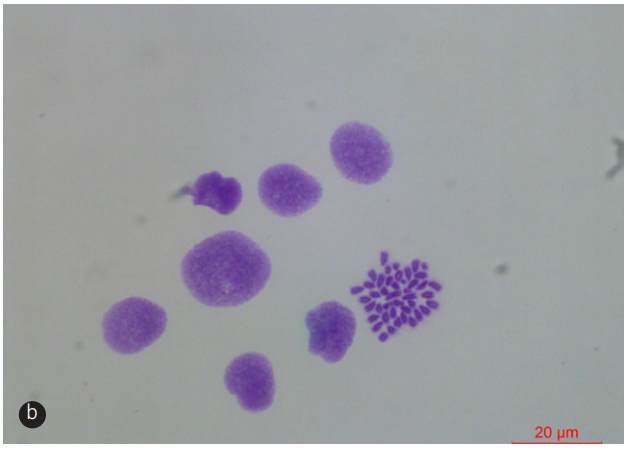
Chromosome analysis

Continuous cell lines often become aneuploid, where one or more chromosomes are missing or present in excess. Determining whether fish cell lines are aneuploid could provide insights into different mechanisms of spontaneous immortalization. Chromosome analyses of a minimum of 100 metaphase spreads per cell line indicated that all our immortalised cell lines display chromosomal aneuploidy, indicative of spontaneous transformation.

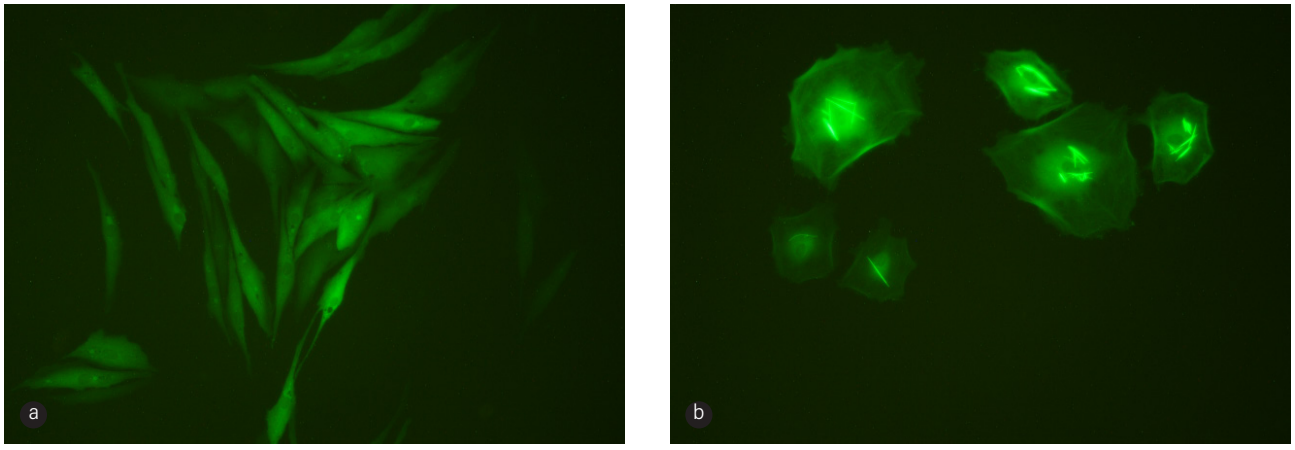
Gene expression studies

Fish cell lines expressing exogenous genes have promising applications in producing recombinant proteins, gene-function studies, and creating transgenic fish. Transfection with plasmids encoding a gene of interest linked to a reporter, such as green fluorescent protein (GFP), is a key method for studying gene expression and localization. Gene expression studies using GFP reporter vectors, like pcDNA3-EGFP and pMAX-GFP, showed high transgene expression in most of our cell lines which confirm that our cell lines are effective *in vitro* systems for evaluating promoter efficiency, intracellular signaling and gene expression.





Metaphase spreads from cell lines showing chromosome numbers



Expression of GFP reporter gene after transfection by Nucleofection in (a) Fibroblast and (b) Epithelial cells

Screening for mycoplasma

Mycoplasma testing is an essential part of cell line quality control. Mycoplasma comprises various species with in the Mollicutes, and cell culture laboratories have long been vigilant about mycoplasma contamination due to its ability to infiltrate cultures without causing visible changes. Over the years, several detection methods for mycoplasma in animal cell lines have been established including culture tests, polymerase chain reaction (PCR), and fluorescent DNA staining. All our cell lines have been confirmed to be mycoplasma-free by screening using the MycoFluor™ Mycoplasma Detection Kit.

Applications of marine fish cell lines in research

Toxicology and environmental monitoring

Marine fish cell lines are an ideal model for toxicity studies since they provide a controlled environment for assessing the effects of environmental pollutants. By exposing these cell lines to contaminants, researchers can determine cytotoxicity, genotoxicity, and mutagenic effects at the cellular level. This data can then be used to gauge the impact on the overall marine ecosystem and guide environmental policies.

Fish disease research

Fish diseases, particularly in aquaculture, pose significant challenges to marine farming operations. Viral and bacterial infections can lead to mass mortality and economic losses. Cell lines derived from fish serve as crucial models for studying the pathogenesis of infectious agents. These *in vitro* studies help in understanding host-pathogen interactions and developing vaccines and therapeutics, significantly advancing fish health management. The establishment of robust and susceptible fish cell lines is essential for isolating and propagating infectious viruses from fish. Cell lines facilitate *in vitro* studies on viral replication and host-pathogen interactions to viral infections under controlled conditions. This knowledge is crucial for developing effective viral vaccines

Genetic engineering and functional genomics

Marine fish cell lines have become indispensable tools in the field of genetic engineering, offering researchers the ability to study gene function, expression, and regulation in a controlled environment. These cell lines enable the manipulation of genes to investigate their roles in growth, stress responses, and immune function, which are critical areas of focus for the advancement of aquaculture. Through techniques like transfection, CRISPR-Cas9, and gene editing, marine fish cell lines provide the foundation for developing transgenic fish, improving disease resistance, and enhancing environmental tolerance, all of which hold great promise for the future of sustainable aquaculture and marine conservation efforts.

Biodiversity conservation

Cryopreservation of marine fish cell lines offers a valuable approach to preserving the genetic diversity of fish species, particularly those at risk due to overfishing, habitat degradation, or climate change. Establishing a "cell bank" allows researchers to store genetic material for future research or potential repopulation initiatives. These preserved cell lines serve as a critical resource for conserving biodiversity and could play important role in reviving species or populations.

Cell based seafood research

The concept of producing laboratory-grown seafood through fish cell and tissue cultures is gaining importance as a solution to the challenges posed by industrial aquaculture and marine capture fisheries. Cell based seafood offers a sustainable alternative to traditional seafood production, addressing key challenges in food security, environmental sustainability, and ethical consumption. This approach would reduce reliance on wild-caught fish, contributing to the conservation of marine ecosystems and biodiversity, especially as global fish stocks decline due to overfishing and climate change. To advance cell-based seafood, more research is needed on fish muscle cultivation, serum-free media formulations, biocompatible edible scaffolds, and bioreactor designs for large-scale production. Although cultivated meat science is still emerging, with the first lab-grown hamburger debuted in 2013, cost remains a significant barrier. In 2020, Eat Just Inc. made a breakthrough with regulatory approval for lab-grown chicken nuggets in Singapore, the first cultivated meat approved for human consumption. However, solid research is crucial for informed decisions based on science rather than speculation.

Conclusion

Marine fish cell lines have proven to be invaluable in addressing a variety of scientific and industrial challenges. In environmental toxicology, for example, these cell lines are used to assess the impact of pollutants, such as heavy metals, pesticides, and oil spills, on marine life. They enable researchers to study the cytotoxic and genotoxic effects of these substances at the cellular level, providing data that can be used to protect marine ecosystems and ensure the sustainability of marine resources. In aquaculture, cell lines help in understanding disease mechanisms, improving fish health management, and developing vaccines and therapeutics to control pathogens that threaten farmed fish populations. Marine fish cell lines are also key to unlocking the potential of marine biotechnology. With the application of advanced technologies like CRISPR-Cas9 for gene editing and omics approaches (genomics, proteomics), the depth of knowledge that can be gathered from these cell lines is rapidly increasing. From environmental monitoring to disease management and biodiversity conservation, they also offer a controlled, reproducible, and ethical method of studying marine life.

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