Developmental and Comparative Immunology xxx (2017) 1–7

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

# Engineered cell lines for fish health research

Bertrand Collet*, Catherine Collins, Katherine Lester

*Marine Scotland, Marine Laboratory, Aberdeen, UK*

ARTICLE INFO

*Article history:*
Received 22 November 2016
Received in revised form
13 January 2017
Accepted 16 January 2017
Available online xxx

*Keywords:*
Cell line
Reporter
Genetically engineered
Over-expression
Gene silencing

ABSTRACT

As fish farming continues to increase worldwide, the related research areas of fish disease and immunology are also expanding, aided by the revolution in access to genomic information and molecular technology. The genomes of most fish species of economic importance are now available and annotation based on sequence homology with characterised genomes is underway. However, while useful, functional homology is more difficult to determine, there being a lack of widely distributed and well characterised reagents such as monoclonal antibodies, traditionally used in mammalian studies, to help with confirming functions and cellular interactions of fish molecules. In this context, fish cell lines and the possibility of their genetic engineering offer good prospects for studying functional genomics with respect to fish diseases. In this review, we will give an overview of available permanently genetically engineered fish cell lines, as cell-based reporter systems or platforms for expression of endogenous immune or pathogen genes, to investigate interactions and function. The advantages of such systems and the technical challenge for their development will be discussed.



## 1. Introduction

Fish immunology is a relatively recent discipline, with advances in knowledge and understanding delayed historically compared to mammalian immunology, but now expanding rapidly in line with the genomic revolution. Due to the delay in the development of fish immunology, and because its research community is small compared to the medical research community, there is a lack of basic tools such as monoclonal antibodies (mAb), capable of separating different immune cells by flow sorting. Well characterised mAb reagents for fish immune cells or molecules are very scarce. For example, a mAb against salmonid CD8 was only recently developed (Takizawa et al., 2011) whereas its mammalian counterpart was isolated over 3 decades ago (Thomas et al., 1980) and made available through commercialisation very soon after. Gene expression analysis by semi quantitative RT-PCR or qPCR was, and often still is, the only method available to monitor changes in gene expression in fish cells to host and non-host signals and stimuli, and as a result is used to its very limit in interpreting molecular responses and interactions.

In addition, the discipline of fish immunology is driven by the global growth in aquaculture of a variety of fish species, belonging to different systematic classes. With fish being recognised as the most diverse group of vertebrate (Ravi and Venkatesh, 2008), it is not surprising that reagents developed for different fish species often do not cross-react between them. The consequence of this is further fragmentation of an already small research community.

A small number of immortal fish cell lines have been empirically isolated over the years from a number of fish species and are used as material to grow viral pathogens, to diagnose viral diseases and as *in vitro* experimental tools (Lakra et al., 2011). However, very little effort has been dedicated to the functional characterisation of these and towards developing genetic engineering methodologies. This is despite the fact that genetically modified fish models, apart from zebrafish, are difficult to obtain and maintain in terms of their generation times for back crosses, and their size and husbandry requirements. In addition salmonid fish have undergone two full genome duplication events during their evolutionary history, making genome manipulation more difficult. Some of these difficulties with the more commercial fish species, may soon be overcome with the advent of CRISPR technology. Nevertheless, genetically engineered cell lines still have great potential in advancing research in fish disease and host response.

The generation of a stable cell line is an essential step for gene function studies (Grimm, 2004). Many of the studies utilising genetically modified cell lines have been based on the transient transfection of the cells which may not reflect the natural expression profile (Gibson et al., 2013; Adamson et al., 2011). However,

* Corresponding author.
*E-mail address:* Bertrand.Collet@gov.scot (B. Collet).

http://dx.doi.org/10.1016/j.dci.2017.01.013
0145-305X/

with progress in gene delivery methods (Schiøtz et al., 2011) there are now examples of genetically modified stable fish cell lines used in fish disease research. The earliest is the stable modification of a goldfish cell line (Isa and Shima, 1987) where *gpt*, a gene encoding for GlcNac-1-Phosphate Transferase (GPT) and conferring resistance to mycophenolic acid (MPA), was integrated into the genome. The following year stable Chinook salmon (*Oncorhynchus tshawytscha*) embryo (CHSE) and a rainbow trout (*Oncorhynchus mykiss*) hepatoma (RTH) cell lines were successfully generated, integrating the G418 resistance gene in both cases (Helmrich et al., 1988). In this review, we will focus on genetically engineered stable fish cell lines that have been characterised and used in either basic or applied research in the fields of fish diseases and fish immunology. This article will provide an update to information in a 2008 review on genomic tools for gene function analysis in salmonids (Martin et al., 2008).

## 2. Promoter -reporter systems

Promoter based reporter systems consist of a gene promoter sequence linked to a reporter molecule, the latter being easily detected and measured when the promoter is activated and the reporter gene expressed. Detection of the molecule can be direct, such as green fluorescence protein (GFP) or its derivatives, or indirect, where an enzyme is expressed and then generates a measurable detectable response in the presence of its specific substrate e.g. luciferase which emits light on cleaving its substrate luciferin.

The promoter sequence to be used in the assay is chosen to demonstrate activation of its native gene, or the biochemical pathways in which the gene is involved.

While there are numerous stable cell reporter assays for different mammalian cell pathways, and for fish pathways targeting detection of environmental or pharmacological toxicants, there are few stable cell reporter systems in fish relating to study of fish disease or pathogens. These fish reporter cell lines have however been used to help demonstrate evolutionary conservation in immune function in lower vertebrates and basis for virulence of fish viral pathogens, and in applied uses in monitoring viraemia dynamics during viral infection in fish. These reporter systems are for the most part based on the type I interferon (IFN) innate antiviral pathway and the type II IFN (γIFN) adaptive immunity.

Though transient cell reporter systems for type I IFN had previously been generated in a zebrafish (Altmann et al., 2004), and in a Chinook salmon embryo (CHSE-214) cell line (Johansen et al., 2004), the first stable fish cell promoter based system for the type I IFN pathway was generated by Collet et al. (2004). The authors transfected the rainbow trout gonad cell line (RTG-2) with a plasmid expressing the firefly luciferase gene under the control of the *mx1* antiviral gene promoter, and selected for a stable, clonal, reporter cell line, named RTG-P1 (ATCC-2829). The MX1 protein is an end effector antiviral molecule in the interferon type I antiviral pathway. It interferes with the transport of viral proteins and the formation of viral particles (Haller and Kochs, 2002). The RTG-P1 cell line was shown to be positive for luciferase activity, i.e. activation of the *mx1* promoter, following exposure to polyinosinic:polycytidylic acid (poly I:C), viral haemorrhagic septicaemia virus (VHSV) and corresponding conditioned media, all known activators of the IFN pathway in mammals. Therefore the RTG-P1 proved a useful tool for detection of IFN, which at the time of RTG-P1 generation, was not isolated in fish. In addition, luciferase activity was seen to increase with increasing dose of poly I:C to a given threshold after which it plateaued, indicating some capacity for IFN quantification.

In 2007, Jørgensen et al. described the establishment of a stable clonal CHSE-214 reporter cell line (CHSE-Mx10) for IFN, with the same reporter construct used to generate RTG-P1. The reporter assay was induced following exposure to Atlantic salmon recombinant IFN-α/β and trout γIFN- in a dose-dependent manner. It did not respond to stimulation with recombinant TNF-α nor recombinant IL-1β.

A stable reporter clonal cell line (RTG-3F7) has also been generated for detection of γIFN activity. In mammals γIFN is a key cytokine in innate and adaptive immunity against viral and intracellular bacterial infections and is produced by T lymphocytes (Schroder et al., 2004). The RTG-2 cell line was stably transfected with a reporter plasmid containing a promoter region of the γIFN inducible gene Transporter of Antigenic Peptides (TAP2) linked to the firefly luciferase gene (Castro et al., 2010). Transient transfections were first used to test promoter regions from three γIFN inducible genes: γIP, LMP2 and TAP2, before selecting the latter for stable cell line development. The cell line was responsive, in a dose dependent way, to recombinant γIFN, and conditioned cell culture supernatant from cells incubated with the γIFN inducer Phyto-HaemAgglutinin (PHA).

Collet et al. (2004) also investigated the involvement of the transcription factor NFκB in fish IFN pathways using the RTG-P1 reporter cell line. They demonstrated that inhibition of NFκB by Pyrrolidine dithiocarbamate (PDTC) blocked poly I:C induction of the RTG-P1 reporter activity, indicating that, as in humans (Alexopoulou et al., 2001), MX production through dsRNA IFN signalling is NFκB dependent in teleost fish.

The role of dsRNA in the fish IFN antiviral immune response was further investigated by Poynter and DeWitte-Orr (2015) using the RTG-P1 cell line. Most viruses generate dsRNA during replication and these are recognised as pathogen associated molecular patterns (PAMPS) by internal and external host cell receptors, initiating induction of IFN and IFN stimulated genes (ISGs) (Jacobs and Langland, 1996). Extracellular high and low molecular weight poly I:C and long (1200 nucleotide) and short (200 nucleotide) *in vitro* transcribed dsRNA from VHSV were shown to activate the RTG-P1 reporter system, with the longer molecules generating a stronger activation, as also observed in mammalian systems (DeWitte-Orr et al., 2009).

Matsuo et al. (2008) used transiently and stably transfected fugu (*Takifugu rubripes*) IFN reporter RTG-2 cell lines to study the function of the Toll like receptor (TLR) TLR22. TLR22 is found only in aquatic animals, and its function at the time of the study was unknown. The reporter system this time consisted of the fugu IFN promoter linked to luciferase, and the RTG-2 cell line was chosen due to its low constitutive and endogenous induced expression of TLR22. Details on whether the stable cell lines were generated from batch culture or clonal culture are not provided. The stable fgIFN reporter cell line (RTG (LucfgIFN)) was transiently transfected with fgTLR22, stimulated with poly I:C and activation of the IFN reporter system measured. It was found that transient expression of the fgTLR22 on the surface (determined by immunohistochemistry) of RTG (LucfgIFN) increased activation of the IFN pathway compared to controls, indicating that TLR22 plays a role in extracellular dsRNA activation of the IFN pathway.

As well as evolutionary functional studies on the activation of the IFN pathway in lower invertebrates, the RTG-P1 reporter cell line has also been used to help understand IFN activation in more applied aspects of fish disease control. DNA vaccines developed against a number of fish rhabdoviruses induce a strong type I IFN response (Boudinot et al., 1998) following vaccination. To investigate this response further, the RTG-P1 reporter cell line was transiently transfected with a plasmid expressing the VHSV surface glycoprotein (Acosta et al., 2006). It was found that when mAbs against VHSV were added to the medium, the IFN reporter activity

Please cite this article in press as: Collet, B., et al., Engineered cell lines for fish health research, Developmental and Comparative Immunology (2017), http://dx.doi.org/10.1016/j.dci.2017.01.013