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Applications and potential uses of fish gill cell lines: examples with RTgill-W1

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Abstract Gills are unique structures involved in respiration and osmoregulation in piscinids as well as in many aquatic invertebrates. The availability of the trout-derived gill cell line, RTgill-W1, is beginning to make impacts in fish health and toxicology. These cells are available from the American Type Culture Collection as ATCC CRL 2523. The cells have an epithelioid morphology and form tight monolayer sheets that can be used for testing epithelial resistance. The cells can be grown in regular tissue culture surfaces or in transwell membranes in direct contact with water on their apical surfaces. The ability of RTgill-W1 to withstand hypo- and hyper-osmotic conditions and their optimal growth capacity at room temperature, make these cells ideal sentinel models for in vitro aquatic toxicology as well as model systems to study fish gill function and gill diseases. RTgill-W1 support growth of paramyxoviruses and orthomyxoviruses like salmon anemia virus. RTgill-W1 also support growth of *Neoparamoeba pemaquidensis*, the causative agent of amoebic gill disease. The cells have been used to understand mechanisms of toxicity, ranking the potencies of toxicants, and evaluating the toxicity of environmental samples. These cells are also valuable for high throughput toxicogenomic and toxicoproteomic studies which are easier to achieve with cell lines than with whole organisms. RTgill-W1 cell line could become a valuable complement to whole animal studies and in some cases as gill replacements in aquatic toxicology.

Keywords Aquatic ecotoxicology · Cytotoxicity · Fish cell culture · Gill cell line · Rainbow trout · RTgill-W1

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Introduction

In vitro models have been invaluable in many areas of life sciences. These experimental systems allow direct access and evaluation of specific functions with higher control of the conditions of assays, reducing variability of responses due to unavoidable stress responses. Ready access to functional cells without the constraints of non-target tissues, provides the possibility for easy studies of cellular mechanisms. However, special considerations must be addressed to establish stable in vitro function. Primary cultures are usually short-lived and require specific culture conditions. Microbial contamination is more common and difficulties in obtaining adequate tissue amounts, have prompted interest in developing permanent cell lines which can provide a much more convenient source of cells. Nevertheless, depending on the question being asked, primary cultures (cells freshly derived from organ of interest) or cell lines (cells that have been passaged many times in vitro) have been used in various aspects of scientific research.

The gills of aquatic organisms are unique organs involved in gas exchange, osmoregulation, and other critical functions (Evans et al. 2005) essential for survival of piscinid and invertebrate species. Thus, damage to gills may reflect in impaired functioning of the organism and eventual death. Studies of gill function have traditionally

been performed in vivo in experimental animals. However, recent advancements in gill cell culture provide an alternate model system to study the gill (Fernandes et al. 1995; Wood and Part 1997; Sandbacka et al. 1999; Fletcher et al. 2000; Wood et al. 2002; Leguen et al. 2007). In these systems, branchial cells are grown on solid or permeable insert supports. Within permeable inserts, gill membranes can be established where experimental manipulations of the apical and basolateral sides can be made once confluent cell layers are formed. This approach allows the activities of ions to be experimentally set on both sides of an epithelium and electrophysiological parameters such as transepithelial resistance and transepithelial potential to be monitored. Reconstructed branchial epithelia withstand prolonged apical exposure to freshwater and show many physiological and morphological characteristics similar to those of the gill epithelium in vivo (Fletcher et al. 2000; Wood et al. 2002; Leguen et al. 2007). However, inherent drawbacks of primary cultures: labor intensive, short life span, and not always easy to obtain in a reproducible and quantitative manner have limited their usage.

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, a gonadal cell line derived from rainbow trout (Wolf and Quimby 1962), mainly for virological studies. In the almost 50 yr 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 (Clem et al. 1996; Bols et al. 2001), toxicology (Babich and Borenfreund 1991; Segner 1998), ecotoxicology (Fent 2001; Castano et al. 2003; Schirmer 2006), endocrinology (Bols and Lee 1991), virology (Wolf 1988), biomedical research (Hightower and Renfrow 1988), disease control (Villena 2003), biotechnology and aquaculture (Bols 1991), and radiation biology (Ryan et al. 2008) are some of the areas in which fish cell lines have made significant contributions.

Many fish cell lines have been derived from dissociated adult or embryonic tissues (Fryer and Lannan 1994) but few have been characterized for tissue of origin with appropriate markers (Bols and Lee 1994). Inasmuch as fish comprise more than half of all vertebrate species together, it is surprising that so few cell lines have been established from piscinid species in comparison to mammals. To date, of the more than 3,400 cell lines deposited at the American Type Culture Collection (ATCC), only 31 cell lines could be found that are of fish origin (see Table 1). Astounding as well, is the fact that although greater than 70% of the earth mass is covered with water and that aquatic organisms prevail with a distinct organ such as the gills, very few cell lines have been initiated from such a unique organ, although a lot of research has been done at the organismal level. Research with cells derived from the gills of fish, a conspicuous organ involved in gas exchange and ionoregulation, have been steady, yet, most of the work involves use of primary cultures despite the reported difficulties for their isolation, maintenance, and reproducibility (Wood et al. 2002; Leguen et al. 2007). 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, from the 31 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.

Of the 12 gill cell lines reported to date (Table 2), none have been described that were initiated from neoplastic or cancerous tissues and only the FG-9307 gill cell line has been reported to undergo spontaneous neoplastic transformation in vitro (Guo et al. 2003). Nevertheless, literature on the use of fish gill cell lines have been scarce and most work performed to date involved two gill cell lines: RTgill-W1 derived from gill explants of adult rainbow trout (*Oncorhynchus mykiss*) (Bols et al. 1994), is representative of gills from freshwater species; whereas, FG-9307, derived from flounder (*Paralichthys olivaceus*) (Tong et al. 1997), is representative of gills from marine fish. In this review, we provide examples of applications and potential uses mainly for the rainbow trout gill cell line RTgill-W1, since RTgill-W1 is readily available from the American Type Culture Collection as ATCC number CRL 2523, unlike FG-9307, which is only available from Dr. Zhang, QingDao, China.

RTgill-W1: Origins, Cell Culture Conditions, and Growth Characteristics

RTgill-W1 is an epithelial cell line derived from the gill explants of normal adult rainbow trout (*Oncorhynchus mykiss*) (Bols et al. 1994). These cells have been authenticated as derived from rainbow trout by microsatellite analysis (Perry et al. 2001). The cells are routinely cultured at room temperature in 75 cm^2 tissue culture flasks with 10 ml of Leibovitz-15 media (L15) with added fetal bovine serum at 10% (*v*/*v*), 100 U/ml penicillin and 100 µg/ml

Table 1. Fish cell lines deposited at the American type culture collection as of September 2008

Fish species	Designation	ATCC no.	Cell type/morphology	Tissue source
Carassius auratus goldfish	CAR	CCL-71	Fibroblast	Normal fin
Clarias batrachus walking catfish	G1B	CRL-2536	Pleomorphic	Gill
Clupea pallasi Pacific herring	PHL	CRL-2750	Epithelial	Larvae
Cyprinus carpio carp	EPC	CRL-2872	Epithelial	Epithelioma papullosum
Danio rerio zebrafish	ZF4	CRL-2050	Fibroblast	Embryo fibroblasts
Danio rerio	ZEM2S	CRL-2147	Fibroblast	Embryo fibroblasts
Danio rerio	SJD.1	CRL-2296	Fibroblast	Caudal fin
Danio rerio	AB.9	CRL-2298	Fibroblast	Caudal fin
Danio rerio	ZFL	CRL-2643	Parenchymal	Normal liver
Fugu rubripes Tora fugu	Fugu eye	CRL-2641	Epithelial	Eye
Fugu niphobles Kusa fugu	Fugu fry	CRL-2642	Fibroblast	Fry
Haemulon sciurus blue striped grunt	GF	CCL-58[a]	Fibroblast	Fin
Ictalurus nebulosus brown bullhead	BB	CCL-59	Fibroblast	CT and muscle
Ictalurus punctatus channel catfish	1G8	CRL-2756	Lymphoblast	Blood cells
Ictalurus punctatus	3B11	CRL-2757	Lymphoblast	Blood cells
Ictalurus punctatus	28S.3	CRL-2758	T lymphoblast	Blood cells
Ictalurus punctatus	42TA	CRL-2759	Macrophages	Blood cells
Ictalurus punctatus	G14D	CRL-2760	T lymphocytes	Blood cells
Ictalurus punctatus	CCO	CRL-2772	Fibroblast	Ovary
Lepomis macrochirus bluegill	BF-2	CCL-91	Fibroblast	Caudal trunk
Morone chrysops white bass	WBE	CRL-2773	Epithelial	Embryo
Oncorhynchus keta chum salmon	CHH-1	CRL-1680	Fibroblast	Heart
Oncorhynchus mykiss rainbow trout	RTH-149	CRL-1710	Epithelial	Hepatoma
Oncorhynchus mykiss	RTG-2	CCL-55	Fibroblast	Fry testis and ovary
Oncorhynchus mykiss	RTG-P1	CRL-2829	Fibroblast	Gonad
Oncorhynchus mykiss	SOB-15	CRL-2301	Epithelial	Liver
Oncorhynchus mykiss	RTgill-W1	CRL-2523	Epithelial	Gill
Oncorhynchus tshawytscha chinook salmon	CHSE-214	CRL-1681	Mixed	Embryo
Pimephales promelas fathead minnow	FHM	CCL-42	Epithelial	CT and muscle
Poeciliopsis lucida topminnow	PLHC-1	CRL-2406	Hepatocyte	Hepatocellular cacinoma
Salmo salar Atlantic salmon	ASK	CRL-2747	Epithelial	Kidney

[a] This cell line is no longer available from ATCC.

streptomycin. Conditions for their routine growth, maintenance, and use in toxicity assays was reported by Dayeh et al. (2005a). The cells exhibit epithelial morphology (Fig. 1) and are believed to have derived from undifferentiated precursor gill stem cells. Given appropriate conditions, mucus-secreting goblet-like cells and cells with abundant mitochondria could be selected (Fig. 2). Additionally, these cells form tight epithelia and withstand exposure to fresh-

Table 2. Fish gill cell lines reported in the literature up to September 2008

Cell line	ATCC no.	Morphology	Species of origin	Reference
G1B	CRL-2536	Pleomorphic	Walking catfish *Clarias batrachus*	Noga and Hartmann 1981
G1	NA	Epithelial	Common carp *Ciprinus carpio*	Chen and Kou 1988
BG/G	NA	Fibroblastic	Bluegill sunfish *Lepomis macrochirus*	Borenfreund et al. 1989
CCG	NA	Epithelioid	Color carp *Ciprinus carpio*	Ku and Chen 1992
ZG	NA	ND	Zebrafish *Danio rerio*	Collodi et al. 1992
RTgill-W1	CRL-2523	Epithelial	Rainbow trout *Oncorhynchus mikiss*	Bols et al. 1994
MG-3	NA	Fibroblastic	Mrigal *Cirrhinus mrigala*	Sathe et al. 1995
RG-1	NA	Fibroblastic	Rohu *Labeo rohita*	Sathe et al. 1997
FG-9307	NA	Epithelioid	Flounder *Paralichthys olivaceus*	Tong et al. 1997
RGE-2	NA	Epithelial	Atlantic salmon *Salmo salar*	Butler and Nowak 2004
RGF	NA	Fibroblastic	Atlantic salmon *Salmo salar*	Butler and Nowak 2004
CF-4	NA	ND	Zebrafish *Danio rerio*	Hogstrand et al. 2007

NA not available, *ND* not described.

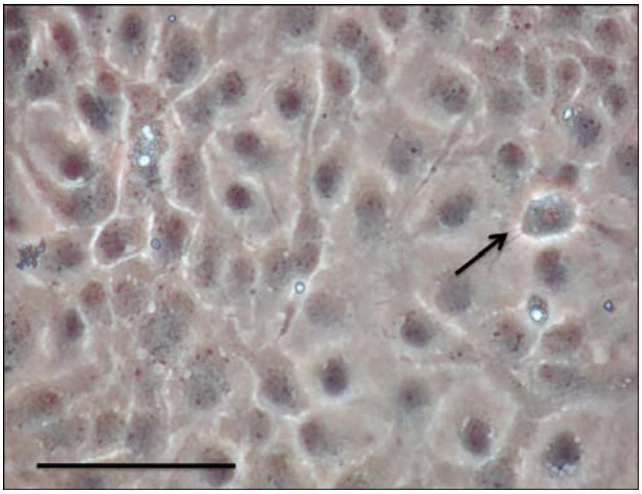

Figure 1. Morphology of RTgill-W1. Phase contrast micrograph of RTgill-W1 monolayer at passage 86. *Arrow* indicates mitotic figure. *Bar*=100 μm.

or saltwater when grown in transwell membrane chambers (Fig. 3). Sandbichler and Pelster (2005) noted that RTgill-W1, like primary trout gill epithelial cells, expressed proteins involved in osmotic stress response when these cells were grown in transwells and were exposed to freshwater on the apical chambers. RTgill-W1 cells are quite tolerant of hypo- and hyper-osmotic conditions and could be grown for prolonged periods in direct exposure with media of varying salinities (Fig. 4). RTgill-W1 are also quite sensitive to cortisol exposure which inhibits cell proliferation and changes cellular morphology (Fig. 5) (Lee, unpublished data).

Gill cell lines in basic research. Most physiological work performed to date involved the use of either perfused gills, primary gill cultures, or whole organisms. Little use of the gill cell lines for basic research have been performed to date. However, Ebner et al. (2007) recently reported using RTgill-W1 to study activation pattern and subcellular distribution of ERK, a mitogen-activated protein kinase; and Krumschnabel et al. (2007) evaluated RTgill-W1 cells for their apoptotic mechanisms, evaluating activation of effector caspases, nuclear condensation, mitochondrial membrane potential, and overall apoptotic volume decrease (cell shrinkage). These authors reported that the RTgill-W1 cells behaved similarly to mammalian cells albeit some differences were noted.

Gill cell lines in fish health research. Fish gill membranes provide a thin entry route to pathogens. Mechanisms of pathogen entry into gill epithelia is poorly understood and although cell lines have been commonly used with mammalian pathogens for mechanisms of host–pathogen relationships, comparatively little usage of gill cell lines have been made for the study of pathogens, except for viruses.

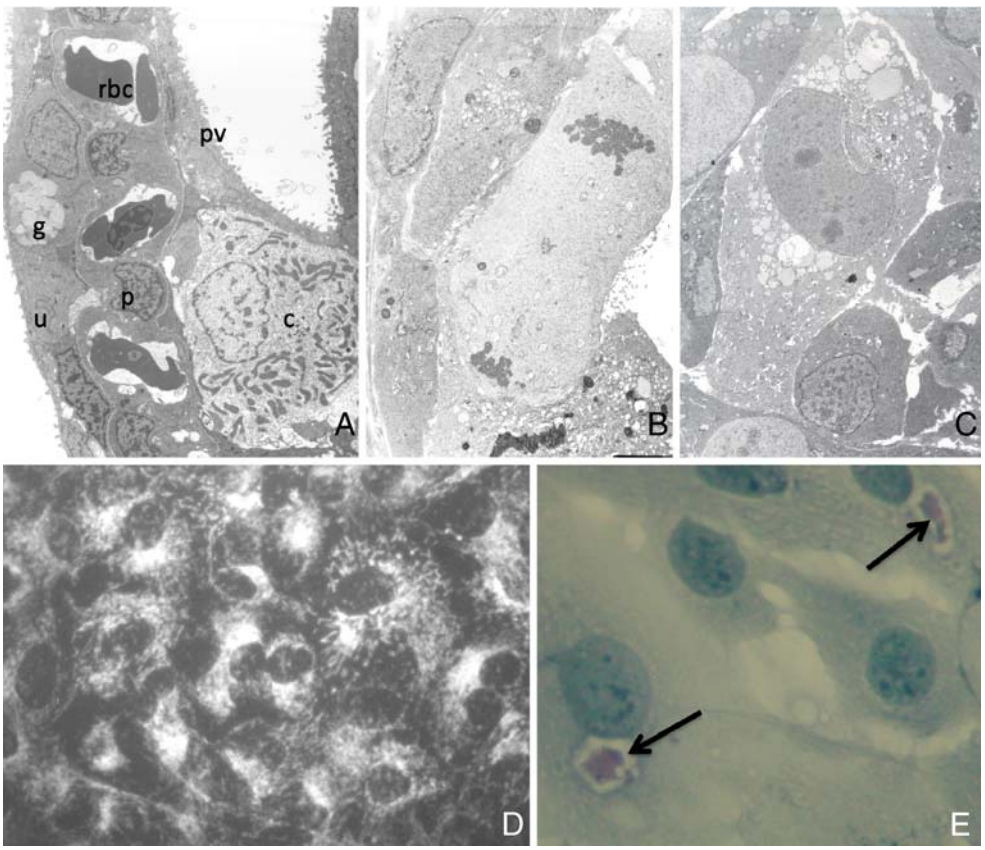

Figure 2. Gill cell types. (*A*) TEM of rainbow trout gill lamella with common cells found at base of lamella: *c* chloride cell, *pv* pavement cells, *g* goblet cell, *u* undifferentiated cell, *p* pillar cell, *rbc* red blood cell. (*B*) TEM of RTgill-W1 monolayer with proliferating cell shown in telophase. (*C*) TEM of RTgill-W1 monolayer with goblet-like cell with coalescing vesicles. (*D*) Fluorescence micrograph of RTgill-W1 monolayer stained with Rhodamine 123 depicting mitochondria rich cells. (*E*) Light micrograph of RTgill-W1 monolayer stained with periodic acid Schiff (PAS) to demonstrate mucopolisaccharide accumulation (*arrows*) in goblet-like cells.

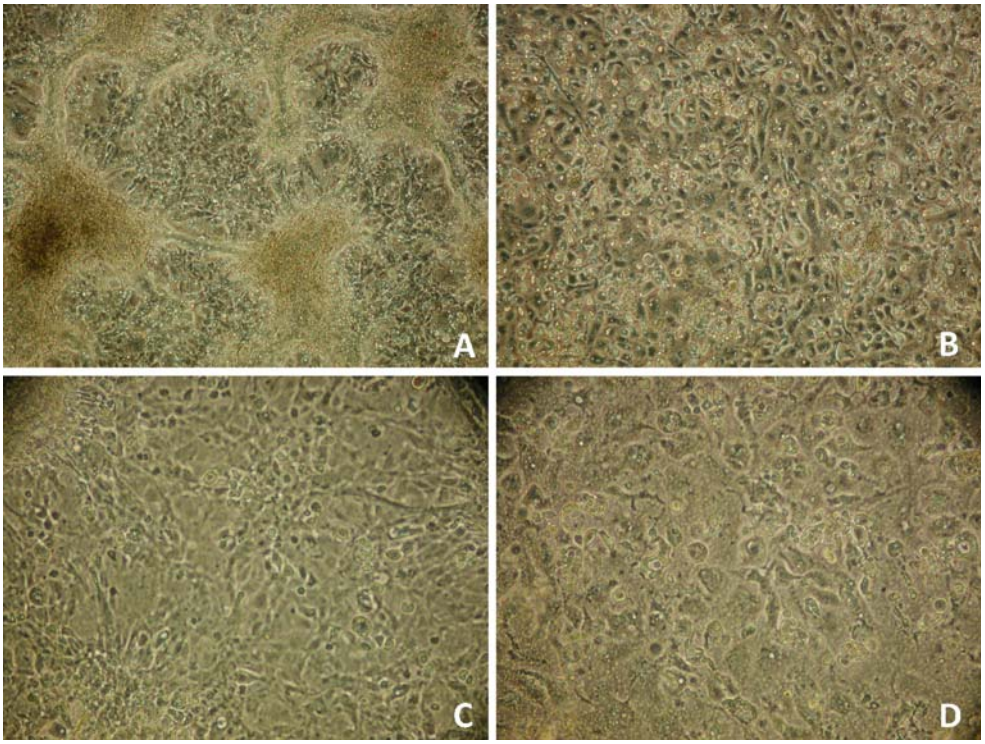

Figure 3. Morphology of RTgill-W1 grown in cell culture inserts for 4 wk with weekly changes of upper and lower chambers. *A*, *C* Freshwater in upper chamber. *B*, *D* Seawater in apical chamber. Lower chambers contained normal L-15 culture media. Mag=×40 for *A*, *B* and ×100 for *C*, *D*.

Several diseases have been reported to affect the gills of fish, among these, viruses have been quite problematic. Viruses, as the ultimate intracellular pathogens, require host cells, and cell lines have been essential in mammalian virology. RTgill-W1 has been shown to support the growth of a novel paramyxovirus isolated from the gills of disease seawater-reared Atlantic salmon (Kvellestad et al. 2003). The complete genome sequence of the virus, dubbed Atlantic salmon paramyxovirus or ASPV, was recently made possible by RTgill-W1's ability to support their growth (Nylund et al. 2008). Characterization of infectious salmon anemia virus or ISAV, an orthomyxo-like virus

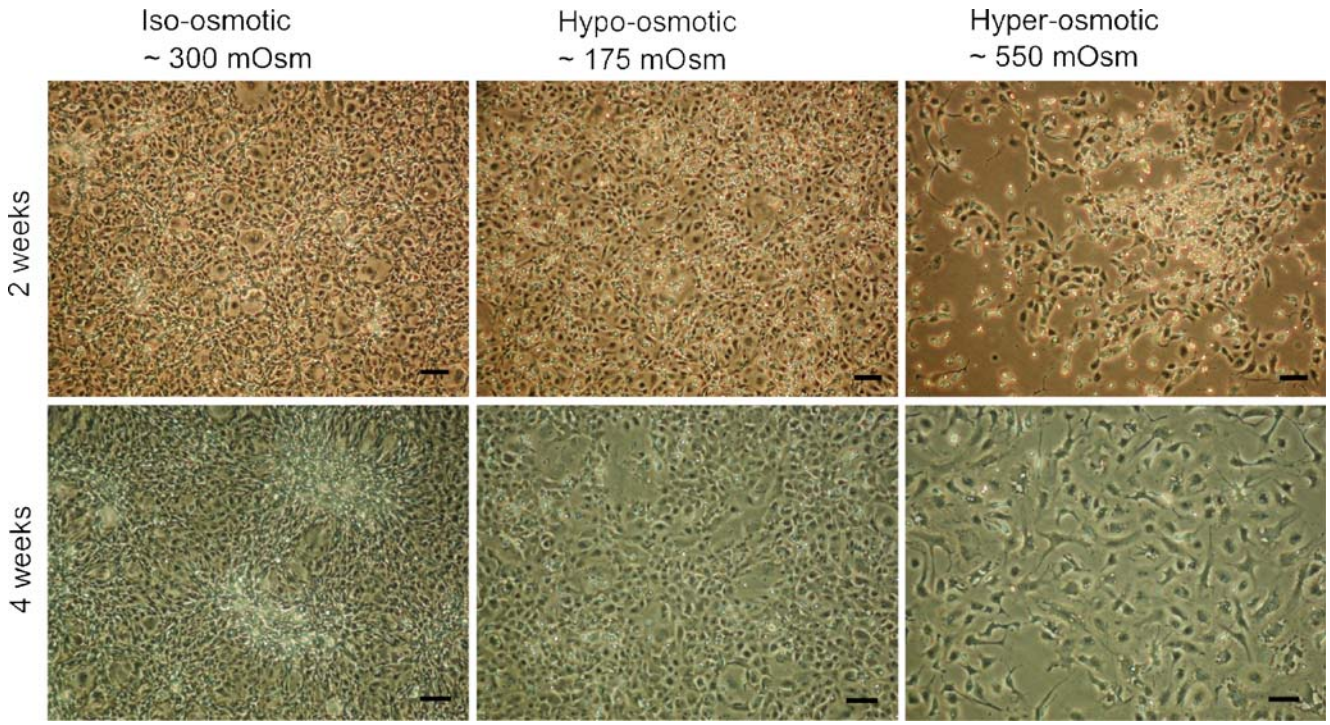

Figure 4. Morphology of RTgill-W1 under varying osmotic conditions. Phase contrast micrographs at ×40. *Bar*=100 μm.

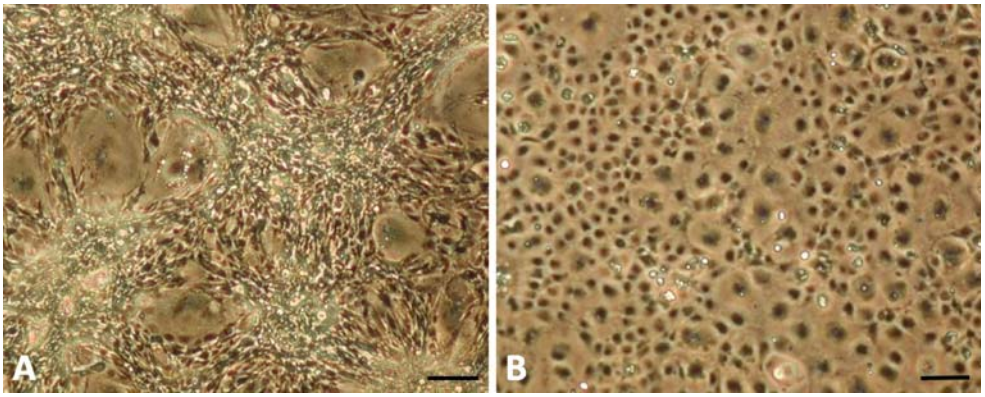

Figure 5. Phase contrast micrographs of RTgill-W1 grown for 21 d in the absence (*A*) or presence of cortisol at 100 ng/ml (*B*). Mag=×40. *Bar*=100 µm.

(Falk et al. 1997), was also made possible by their ability to grow inside RTgill-W1 cells.

While viruses are the ultimate parasites using host cell's mechanisms for reproduction, the study of obligate intracellular bacterial and/or protozoan parasites could also be facilitated with the aid of cell lines. For instance, RTgill-W1 cell line could be useful for studies of gill infecting microsporidia such as *Loma salmonae* (Kent and Speare 2005), the causative agent for microsporidial gill disease of salmonids which is among the most significant infectious diseases affecting aquaculture-raised chinook salmon in Canada (Speare et al. 2007). The usefulness of fish cell lines to study microsporidia is addressed in a separate manuscript by Monaghan et al. (this issue).

The study of ectopic parasites infecting gills could also be investigated using gill cell lines. Noga (1987) used the G1B cell line to study *Amyloodinium ocellatum*, a dinoflagellate commonly found in fish gills and evaluated the effectiveness of an antiprotozoal drug in vitro. The RGE-2 cell line from Atlantic salmon gills (Butler and Nowak 2004), was developed to facilitate study of amoebic gill disease caused by *Neoparamoeba* species. Lee et al. (2006) demonstrated rapid growth and high yield of a lab strain of *Neoparamoeba pemaquidensis* using RTgill-W1 and also demonstrated the specificity of the amoeba for a gill cell line over nine other fish cell lines derived from other tissues. Thus, gill cell lines could be very useful for studying organ-specific pathogens.

Gill cell lines in toxicology research. The gills of aquatic organisms are the primary target and uptake sites of water contaminants (Evans 1987). As such, gills are exquisite organs for the study of aquatic toxicant effects. However, gills in vivo are difficult to evaluate or manipulate, thus, gill cells in vitro could represent ideal systems for the study of aquatic contaminants. While primary gill cultures have been used for the evaluation of aquatic toxicants (Lilius et al. 1995; Pärt 1995; Sandbacka et al. 1999; Wood et al. 2002), the difficulty of their isolation, maintenance, and reproducibility makes them cumbersome to use. Permanent cell lines, on the other hand, are easy to maintain and manipulate and produce highly reproducible results. Thus, the use of fish cell lines in toxicology and ecotoxicology has been relatively broad and several reviews have been published on the topic: Segner (1998); Fent (2001); Castano et al. (2003); Bols et al. (2005); and Schirmer (2006).

Gill cell lines provide an opportunity to study both cytotoxicity and biotransformation of chemicals at the branchial level in much more detail than is possible in vivo. Both, FG-9307 and RTgill-W1 have been used in toxicity testing of various chemicals. For instance, the toxicity of organophosphorous pesticides were evaluated with FG-9307 cells (Li and Zhang 2001; 2002), while RTgill-W1 have been used to evaluate the toxicity of industrial effluents (Dayeh et al. 2002), including petroleum refinery effluents (Schirmer et al. 2001), and have also been used to evaluate the toxicity of polycyclic aromatic hydrocarbons (Schirmer et al. 1998a, b, 1999) and metals (Dayeh et al. 2005b) including Cu, Cd, Zn, Fe, and Ni. Most recently, Bopp et al. (2008) used RTgill-W1 cells to further evaluate toxicity of copper and hypothesized that copper-induced loss in viability and genotoxicity in trout gills may partially be triggered by the generation of reactive oxygen species. Similarly, the toxicity of polibrominated diphenyl ethers (PBDEs) when tested with RTgill-W1 (Shao et al. 2008) as well as with the trout liver cell line RTL-W1, suggest that these compounds mediate cell injury through a mechanism that may involve oxidative stress.

The biggest advantage of using RTgill-W1 though, is that environmental samples can be directly evaluated on these cells. Whole water samples can be evaluated on RTgill-W1 by direct exposure without extraction or concentration steps. The environmental water samples could be added to gill cells for whole effluent testing (Lee et al. 2008). For instance, industrial effluents (Dayeh et al. 2002) or oil-sands process affected waters (Lee et al. 2008), were mixed with L-15 media components and the toxicity of these samples were evaluated using RTgill-W1 cells. The evaluations were done in blind studies without knowledge of sample content, yet the toxicity of the samples correlated with their in vivo toxicity to whole fish (Dayeh et al. 2002)

or to potentially toxic chemical content such as naphthenic acid and salinity content (Lee et al. 2008).

Studies with cultured cells permit the determination of molecular and cellular mechanisms through which pathogens cause disease or pollutants lead to toxic effects in organisms at sublethal and chronic levels. Gill cell lines are proving useful for evaluating the effects of aquatic samples, pathogens, drugs, and toxicants on cellular functions. The availability of the permanent cell line RTgill-W1 is therefore beginning to make major impacts in fish research.

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