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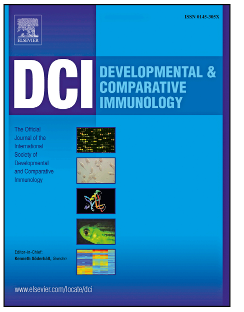

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Extracellular dsRNA induces a type I interferon response mediated via class A scavenger receptors in a novel Chinook salmon derived spleen cell line.

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Abstract (max 150 words) At 150 words

Despite increased global interest in Chinook salmon aquaculture, little is known of their viral immune defenses. This study describes the establishment and characterization of a continuous cell line derived from Chinook salmon spleen, CHSS, and its use in innate immune studies. Optimal growth was seen at 14-18^{o}C when grown in Leibovitz's L-15 media with 20% fetal bovine serum. DNA analyses confirmed that CHSS was Chinook salmon and genetically different from the only other available Chinook salmon cell line, CHSE-214. Unlike CHSE-214, CHSS could bind extracellular dsRNA, resulting in the rapid and robust expression of antiviral genes. Receptor/ligand blocking assays confirmed that class A scavenger receptors (SR-A) facilitated dsRNA binding and subsequent gene expression. Although both cell lines expressed three SR-A genes: SCARA3, SCARA4, and SCARA5, only CHSS appeared to have functional cell-surface SR-As for dsRNA. Collectively, CHSS is an excellent cell model to study dsRNA-mediated innate immunity in Chinook salmon.

Keywords: Chinook salmon, cell line, polyinosinic:polycytidylic acid, interferon-stimulated genes, innate immunity, dsRNA

1. Introduction

Of the Pacific salmon species, Chinook salmon (*Oncorhynchus tshawytscha*) is the largest and most highly valued in North America (Christensen et al, 2018, Ohlberger et al, 2018). As a result, this species has historically been a focus for capture fishery production. Up until 1922, as many as 11 million kg of Chinook salmon were harvested annually before a decline in wild populations was noted. Unfortunately, native populations are in decline at such a rate that annual harvests are now approximately 2 million kg per year (Johnson et al, 2017). This has resulted in Chinook salmon's addition to the threatened species list despite concerted efforts to replenish wild populations via hatchery bred stocks (Knudsen et al, 2006, Paquet et al, 2011). Due to the inability of capture fisheries to meet the rising demands for Pacific salmon consumption, the culture of Chinook salmon is a promising solution to alleviate pressure from wild stocks. As a relatively undomesticated species, a deeper understanding of the Chinook salmon immune system is necessary to ensure successful aquaculture practices.

Viral pathogens present a formidable obstacle in aquaculture due to their rapid replication rate and persistence in the environment (Oidtmann et al, 2017). As every milliliter of seawater contains roughly 10^{7} viral particles (reviewed in Suttle, 2005), interaction with these microorganisms is unavoidable and can be detrimental in aquaculture environments. Fortunately, vertebrates have developed an arsenal of

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pattern recognition receptors (PRRs) designed to recognize and initiate appropriate responses to viral pathogen associated molecular patterns (PAMPs). One such PAMP, double-stranded (ds) RNA, is produced by all viruses at some point during their lifecycle (Jacobs and Langland, 1996, DeWitte-Orr and Mossman, 2010). In response, fish and other vertebrates have evolved several nucleic acid binding PRRs including toll-like receptors (TLRs), RIG-I receptors (RLRs) and class A scavenger receptors (SR-As) to sense dsRNA (reviewed by Poynter et al, 2015a). Binding of dsRNA initiates a signal cascade culminating in the production of type I interferons (IFNs), such as IFN1 in fish, that can induce the expression of interferon-stimulated genes (ISGs) in both a paracrine and autocrine fashion. Many of the resulting ISGs are responsible for interfering with, and rendering host cells non-permissive to, viral infection often referred to as an "antiviral state" (Zhang and Gui, 2012). In fish, significant antiviral activity has been demonstrated for several ISGs including Vig3 (known in mammals as ISG15) and Mx1, but there is still limited information regarding their functional mechanisms (Poynter and DeWitte-Orr, 2016). As antiviral immune responses may vary depending on the host species (Heil et al, 2004, Kuzmann et al, 2017), appropriate model systems are required to further our understanding of viral pathogens and antiviral responses in aquatic species.

Eukaryotic cell lines offer a controlled, cost-effective method to explore antiviral immune function within a single-cell monoculture. Currently, the only cell line available for the study of Chinook salmon is CHSE-214, an embryonic epithelial-like cell line. CHSE-214 is commonly used in comparative virology due to its permissibility in supporting propagation of aquatic viruses (MacDonald and Kennedy, 1979). Interestingly, these cells are incapable of mounting an effective antiviral response when exposed to extracellular dsRNA (Jensen et al, 2002b). When CHSE-214 is transfected with the synthetic dsRNA, polyinosinic:polycytidylic acid (pIC) the innate antiviral IFN response is intact (Jensen et al, 2002b, Monjo et al, 2017), revealing the intracellular signaling pathways are not defective. CHSE-214 cells appear to lack surface receptors for dsRNA as the cells do not bind to extracellular dsRNA, however when a human dsRNA receptor, SR-AI, is expressed in the cells it enables binding capabilities (Monjo et al, 2017). This apparent absence of cell-surface receptors that can uptake dsRNA makes CHSE-214 an excellent model for transfection studies to understand dsRNA sensing. However, as most fish cells can sense extracellular dsRNA, a more biologically equivalent Chinook salmon cell line that uptakes extracellular dsRNA without transfection is desirable.

The present study describes the establishment and characterization of CHSS, an epithelial-like cell line derived from the spleen of a triploid Chinook salmon. Although CHSS presents a diploid phenotype, its creation provides a valuable tool for understanding the innate antiviral immune response in Chinook salmon. Following exposure studies to pIC, CHSS was observed to both take up and subsequently respond to extracellular dsRNA. As CHSS is the only known cell line created from an adult Chinook salmon, these cells are much more relevant for understanding species specific aspects of immunity, physiology, and cellular function.

2. Materials and Methods

2.1 Primary cultures

A 200 g Chinook salmon was retrieved from a net-pen containing a 99-100% triploid population, based on the rate at which maturation was Impaired (John Heath, personal comm.), that was produced and maintained at Yellow Island Aquaculture Ltd (YIAL, Quadra Island, BC, Canada). The fish was euthanized with an overdose of clove oil prior to tissue collection. All procedures were performed following the guidelines of the Animal Care Committee at the University of Waterloo. The spleen, caudal fin and gills were collected and stored in L-15 media supplemented with 20% fetal bovine serum (FBS, Gibco) and 200 U/mL of penicillin and 200 U/mL streptomycin (Thermo Scientific). Upon arrival to the University of Waterloo, each tissue type was diced into small pieces in a laminar flow hood and rinsed three times with Dulbecco's buffered saline solution (DPBS, Lonza) containing the same antibiotics as noted above. Tissue pieces were then transferred to 25 cm^2 flasks (BD Falcon) where approximately 1-2 mL of L-15 supplemented with 20% FBS and the previously described antibiotic concentrations. Primary cultures were established by the explant outgrowth method as previously demonstrated with walleye spleens and fins (Vo et al, 2015ab; 2016). Flasks were then incubated at 18°C with media changes occurring every 2-3 days. Only the primary adherent cells from the spleen were able to outgrow and eventually developed into the CHSS cell line.

2.2 Maintenance of cell cultures

CHSS was routinely grown in L-15 supplemented with 20% FBS at 18°C. Following the first passage, CHSS was subcultured at a 1:2 ratio on a weekly or bi-weekly basis using trypsin (Lonza). CHSS has been maintained for over two years and has undergone over 25 passages. The embryonic Chinook salmon cell line, CHSE-214 was used in this study for comparison. CHSE-214 was routinely grown in L-15 supplemented with 10% FBS at 18°C and was subcultured at a 1:3 ratio every 1-2 weeks using trypsin.

2.3 Cryopreservation of CHSS

Approximately 3 x 10^6 CHSS cells at multiple passages were cryogenically frozen in L-15 containing 20% FBS and 10% dimethyl sulphoxide (DMSO, Sigma). Cells were initially frozen at -60°C overnight and subsequently immersed in liquid nitrogen (-196°C) for long-term storage. To determine cell viability upon thawing, a trypan blue (Sigma) exclusion test was performed using a haemocytometer under a phase contrast microscope (Leica).

2.4 Optimal growth conditions of CHSS

Optimal temperature of growth for CHSS was analyzed between 4 and 26°C. CHSS cells were seeded into nine 6-well plates (Fisher Scientific) at a concentration of 2 x 10^5 cells/well and incubated overnight at 18°C. Three wells of one plate were used to provide the day 0 cell counts. Two 6-well plates were incubated at each of the four temperatures studied (4, 14, 18 and 26°C) for 2 weeks. On days 3, 7, 10 and 14, triplicate wells from each temperature were washed with 1 mL of DPBS and cells were dissociated using 300 µL of trypsin (Gibco). Cell counts for each well were determined using a hemocytometer under a phase contrast microscope (Leica). Cell counts for each time interval were averaged and calculated as a percentage of the day 0 growth to determine the percent growth.

To determine the optimal FBS concentration for maintenance of CHSS, nine 6-well plates were seeded with cells as described above. Following overnight adherence at 18°C, media was removed from all plates and two 6-well plates received 2 mL of either 5% FBS, 10% FBS, 15% FBS or 20% FBS media. Plates were then returned to the 18°C incubator. On days 3, 7, 10 and 14, triplicate wells from each FBS concentration were washed with 1 mL of DPBS so that cells could be dissociated and counted as described above. Statistical analysis for both optimum temperature and FBS concentration was conducted using a two-way ANOVA and Tukey's post-hoc test through the GraphPad Prism software (v7.0, GraphPad Software, Inc. USA)

2.5 Determining ploidy of CHSE-214 and CHSS

Although CHSS was believed to be created from triploid fish tissue, it was necessary to confirm this assumption through flow cytometric analysis. As a positive control for the diploid karyotype, the ploidy of CHSE-214 (McCain, 1970) was also examined. All cells analyzed were grown to 60% confluency and a media change was performed two days prior to flow cytometric analysis. Single cell suspensions were prepared in 500 µL of DPBS following cell detachment by trypsin-EDTA and two rinses with ice-cold DPBS. Seven milliliters of ice-cold 70 % ethanol was used as a fixative and added drop-wise to the cell suspensions while simultaneously vortexing. Fixation was incubated at 4 °C for 30 min. Fixed cells were pelleted at 800 g for 5 min, rinsed once with 5 mL of ice-cold PBS and resuspended in 500 µL of 50 µg/mL RNAse A at 37 °C for 15 min (as performed by Reiger and Barreda, 2016). All contents were transferred to individual 5-mL BD Falcon polystyrene round-bottom test tubes and were then brought to a final volume of 1 mL with ice-cold DPBS. Twenty microliters of 1 mg/mL propidium iodide (Invitrogen) was added to the cell suspensions and incubated at 37 °C for 15 min. DNA content assessment was performed with the FACSAria Fusion Cell Sorter with an integrated 3-laser and 9-color detection unit (BD Biosciences). Data analysis was completed using the FlowJo® software (https://www.flowjo.com).

2.6 Genetic Analyses

2.6.1 Validating species of origin

Genomic DNA was extracted from CHSS cell pellets using the DNeasy Blood and Tissue Kit (Qiagen) as described by the manufacturer. To validate the origin of the CHSS cells, primers were designed to amplify a segment of the Chinook salmon mitochondrial cytochrome c oxidase subunit (cox1, **Table 1**). The PCR parameters used were as follows: a 5-min denaturation step (95^{o}C), followed by 35 cycles of a 30-s denaturation step (95^{o}C), a 30-s annealing step (52^{o}C) and a 30-s extension step (72^{o}C), followed by a final extension of 5 min. The PCR product was then run on a 1.5% agarose gel with 1X GelGreen (Biotium). Bands of the desired size were cut out of the gel and extracted using the QIAquick Gel extraction kit (Qiagen). The resultant fragments were cloned into pGEM®-T Easy plasmids (Promega Corporation) and transformed as previously described by Semple et al, 2018. Five colonies per PCR reaction were selected and grown in LB medium. Plasmids were extracted from the transformants using the GenElute Plasmid Miniprep Kit (Millipore Sigma) according to the manufacturer's instructions. Plasmids were sequenced using SP6 primers at the TCAG sequencing facility (Sick Kids Hospital, Toronto, Ont.). The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6.2 Microsatellite analysis

Because CHSS was observed to have a similar morphology to the long-term CHSE-214 cell line, microsatellite analysis was used to confirm that these cell cultures originated from separate individuals. Genomic DNA was extracted from both CHSS and CHSE-214 cell pellets as described above. Individual genotypes were determined through PCR at 10 previously described microsatellite loci, specifically Oneu8, Oneu14 (Scribner et al. 1996), Omm1135 (Rexroad et al. 2001), Omy325 (O'Connell et al. 1997), OtsG432, OtsG474 (Williamson et al. 2002), Ots1, Ots4 (Banks et al. 1999), Ogo4 (Olsen et al. 1998) and Ssa85 (O'Reilly et al. 1996). PCR conditions included: a 5-min denaturation step (94°C), followed by 30 cycles of a 20-s denaturation step (94°C), a 20-s annealing step (64.6^{o}C – Ssa85, Omy325, Ots4; 63.5^{o}C – Oneu8, Ogo4; 54.3^{o}C – Oneu3; 58.3^{o}C – Omm1135, Ots1; 60.2^{o}C – OtsG474, OtsG432) and a 30-s extension step (72 °C), followed by a final extension of 3 min. All PCR reactions yielded single band products which were then excised and gel-extracted as described above. Subsequent cloning and sequencing of at least 10 clones per loci for each cell culture was completed as described above.

2.6.3 MH class I Genotyping of CHSS and CHSE-214

Primers and PCR conditions as described by Lehnert et al (2016) were used to determine the MH class I genotypes of CHSS and CHSE-214. The primers selected ensured amplification of the peptide binding groove at the α1 domain in Chinook salmon. Two PCR reactions were performed per cell line and at least 26 clones from each PCR reaction were sequenced. PCR product purification, cloning and subsequent sequencing was conducted as described above. The resultant allele sequences were aligned using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/).

2.7 Assessing the antiviral response of CHSS following stimulation with dsRNA

2.7.1 Effect of poly I:C on the viability of CHSS

To confirm that pIC does not influence the survival of CHSS, a viability assay using Alamar Blue was conducted. Cells were plated in 24-well plates at a density of 1 x 10^5 cells/well and allowed to adhere overnight. The following day, media was removed from all wells and, in replicate wells of four, was replaced with 20% FBS media containing either 1 μg/ml, 10 μg/ml or 100 μg/ml of pIC (Sigma Aldrich, P1530). pIC from Sigma Aldrich is heterogenous in length but we have previously shown that it had an average size of 500 bp (Poynter and DeWitte-Orr, 2017). Four wells received 20% FBS media alone to represent the no pIC control.

After 24 hours, media was removed from the sample wells and 400 µl of Alamar Blue (Invitrogen) working solution (PBS with 5% Alamar Blue) was added to each well. The 24-well plate was incubated in the dark at RT for one hour and the fluorescence of each well was measured with an excitation of 520 nm and an emission at 590 nm using the Synergy H1 Hybrid Multi-Mode Reader (BioTek). The relative fluorescence units (RFUs) were used to determine the viability of the cells.

2.7.2 dsRNA-induced expression of antiviral genes by quantitative Real-time RT-PCR (qRT-PCR)

In 6-well tissue culture plates, CHSS was seeded at 1.45 × 10^6 cells/well. The cells were exposed to 1 or 10 µg/mL pIC in 2 mL of L-15 with 2 % FBS. Negative control cultures received no pIC. Trypsin-EDTA was used to collect cells at 3 h and 6 h after pIC exposure and cell pellets were frozen at -80 °C. Total RNA was extraction and cDNA synthesis was completed as described above. All cDNA samples were diluted 1:10 prior to qRT-PCR measurement. The primers for salmonid IFN1, Mx1 and Vig3 used for qRT-PCR were previously validated for CHSE-214 cells as described recently by our group (see **Table 1**). All qRT-PCR reactions contained: 2.5 µL of 1:10 diluted cDNA, 5 µL of LightCycler® SYBR® Green Master Mix (Roche), 2.5 µL of 0.2 µM forward and 0.2 µM reverse primers. qRT-PCR was run using the LightCycler® 480 Instrument II and LightCycler® 840 SW 1.5.1 analysis software (Roche). The thermocycling program was 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 55 °C for 5 s and 72 °C for 8 s, followed by 60 °C for 10 min. Controls containing RNA alone were included for each plate to account for possible contaminating genomic DNA. Data was acquired using the ΔΔCt method. Relative gene expression was normalized to the reference gene (β-actin) and expressed as fold change with respect to the control group. Three independent experiments were performed. Statistical analysis was conducted using a two-way ANOVA through the GraphPad Prism software (v7.0, GraphPad Software, Inc. USA)

2.7.3 Effect of blocking SR-A ligand binding on extracellular dsRNA-induced IFN and ISG expression

Polyinosinic acid (pI) is a well-characterized SR-A competitive ligand while polycytidylic acid (pC) is its SR-A non-competitive ligand counterpart. Because both pI and pC are nucleic acids like pIC, they were used in this experiment. In 6-well tissue culture plates CHSS was seeded at 1.5×10^6 cells/well. The cells were pre-treated with 250 µg/mL pI or pC (Sigma Aldrich, P4154 and P4903 respectively) for 30 min. Poly IC was then directly dosed into the wells so that the its final concentration was 1 µg/mL and cultures were incubated for 3 h prior to RNA extraction and qRT-PCR as previously described. There were four controls: negative control received no exogenous nucleic acid additive, one control received only pI, one culture received only pC, and the last control received pIC. Three independent experiments were performed. Statistical analysis was conducted using a two-way ANOVA through the GraphPad Prism software (v7.0, GraphPad Software, Inc. USA).

2.7.4 Cell associated dsRNA assay and epifluorescence microscopy

pIC was fluorescently labeled with Alexa Fluor™ 488 using the Ulysis™ nucleic acid labeling technology (Thermo Fisher Scientific). Excess unbound Alexa Fluor™ 488 dye was removed using the Micro Bio-Spin™ P-30 Gel Columns (BioRad). Fluorescently labeled pIC was diluted in the growth medium at 50 µg/mL and added into a 4-chamber cell culture slide (BD Falcon) where 2×10^5 CHSS cells had been previously seeded per well. Control cultures received the same growth medium but without Alexa Fluor™ 488 labeled poly IC. After 1 h incubation at 21°C, cells were washed three times with PBS, fixed with 3.7 % formaldehyde for 15 min, rehydrated with one wash of PBS and air-dry for 10 min. The slide was mounted on the DAPI-containing Fluoroshield medium (Sigma Aldrich). Epifluorescence was visualized with the Nikon Eclipse Ti-S fluorescence microscope; fluorescence images were acquired and analyzed with the NIS-Elements software.

2.8 Presence of Class A Scavenger Receptor (SR-A) transcripts in CHSE-214 and CHSS

RNA was extracted from CHSS and CHSE-214 cell pellets containing $1.5 – 3 \times 10^6$ cells using the RNeasy Mini Kit (Qiagen) and subsequently treated with DNase I (Thermo Scientific) as described by the manufacturers. The Synergy H1 Hybrid Multi-Mode Reader and the Gen5™ Data Analysis software (BioTek) were used to acquire RNA concentrations on the Take3™ Micro-volume plate (BioTek) and to

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check RNA purity. Complementary DNA (cDNA) was then synthesized from 500 ng of RNA using a qScript cDNA SuperMix (Quantabio) as described by the manufacturer.

PCR reactions were subsequently used to amplify scavenger receptor sequences from cDNA. Reactions contained 1X Phusion High-Fidelity master mix (Fisher Scientific), 0.5 µM forward primer, 0.5 µM reverse primer (presented in **Table 1**), 2 µl of cDNA, and up to 40 µl with molecular biology grade water. Reactions were run in a Bio-Rad T100 thermal cycler (Bio-Rad) using the following conditions: 98°C 30-s, 34 cycles of 98°C 10-s, T_a (see **Table 1**) 10-s, 72°C 30-s followed by 72°C for 5-min. Reactions were run on a 1% w/v agarose gel in TAE buffer and products were isolated and purified using the GenepHlow Gel/PCR Kit (FroggaBio). Products were sequenced at the University of Guelph Genomics Facility Advanced Analysis Centre (Guelph, ON). Sequences were compared to the known corresponding rainbow trout sequences using EMBOSS Needle sequence pairwise alignment.

3. Results and Discussion

3.1 Development and characterization of the CHSS cell line

In the present study, CHSS, an epithelial-like cell line derived from the spleen of a Chinook salmon, was developed and its capacity to respond to extracellular dsRNA was assessed. Initially, cellular outgrowth from the spleen tissue was very slow with cells presenting a fibroblastic-like morphology (**Figure 1A**). Repeated episodes of cellular deterioration occurred in primary cultures initiated from the tissue explants. After one year in culture, progeny cells started to proliferate in patches of colonies in the flask (**Figure 1B**). Early subcultures by trypsin still resulted in significant loss in adherent cells. Survivor cells presented two consistent cellular morphologies, epithelial-like and fibroblastic-like (**Figure 1C**). Following subsequent passages, the epithelial-like morphology became predominant and gave rise to the CHSS cell line (**Figure 1D**). CHSS has been maintained in culture for over two years and has undergone over 25 passages.

Once established, it is essential to determine the optimal growth conditions of novel cell lines to ensure that both preliminary and future studies are completed reproducibly. CHSS displayed the highest growth rate at 14 and 18°C with no significant differences between these two temperatures (**Figure 2A**). The doubling times for the optimal temperatures of 18°C and 14°C were 92.4 hours and 97.6 hours respectfully. Depending on geographical origin, adult Chinook salmon are reported to have a preferred temperature range between 12-15°C (Bell, 1986, Richter and Kolmes, 2005) but optimal growth of this species is seen at 15-19° depending on feeding rates (Brett et al, 1982, Marine, 1997). As CHSS was derived from an adult Chinook salmon, an optimal growth temperature of 14-18^{o}C fits within the anticipated thermal optimum for this species as well as with other relevant *in vitro* models.

When characterizing media supplementation and long-term storage, CHSS displayed the greatest proliferation in L-15 media supplemented with 20% FBS at 18^{o}C (**Figure 2B**). As there was no significant difference in cell growth for the first week between 20% and 15% FBS, growing CHSS at the lower FBS concentration would be a cost-effective alternative pending consistent passaging of the culture. The doubling times for the optimal FBS concentrations of 20% and 15% were 101.9 hours and 123.8 hours respectfully. CHSS was also able to withstand cryogenic storage presenting on average 75% viability upon thawing (data not shown). Thus, it appears that CHSS can thrive long-term in an *in vitro* culture system and as such can be used as a Chinook salmon cellular model.

3.2 Ploidy analysis of CHSS

CHSS originated from a Chinook salmon selected from a 99-100% triploid population. Despite this, flow cytometric analysis suggested that CHSS was not triploid and contained a similar DNA content to that of the nearly diploid cell line CHSE-214 (previously shown by Philippon-Fried in 1979) (**Figure 3**). This reflects an unpredictable and unavoidable phenomenon observed in rapidly growing cell lines where genomic rearrangements and chromosome instability occurs (Vcelar et al, 2018). It is unclear why CHSS cells did not retain triploidy in culture. There might be a very minute chance that the fish that gave rise to CHSS was in the 1% percentile diploid of the 99-100% triploid population. But the fish had a growth pattern and biology of a typical triploid Chinook salmon. A possible explanation for the extensive chromosome loss could be due to the consequence of repeated cycles of cell growth and death observed during the early passages of CHSS. This process may have selected for the survival of cells that successfully underwent unequal mitosis, prophase chromosome reduction or reductional grouping to produce a stable karyotype, similarly to what has been described in other eukaryotic cell cultures (Nuti Ronchi et al, 1992, Gerstein et al, 2008). As this would take many replication cycles, this could also explain the extended time required to establish CHSS.

Even though a large number of salmonid cell lines have been successfully developed in the past five decades, none of them are triploid cell lines or described as from triploid fish. This feat has been accomplished in two other teleostean species, the oriental weather loach (*Misgurnus anguillicaudatus*) (Li et al, 2015) and the olive flounder (*Paralichthys olivaceus*) (Peng et al, 2016). The original research regarding the establishment of these cell lines does not discuss any developmental issues comparable to what was experienced during the early passages of CHSS. This discrepancy could be related to differences in the species, their genome size and their cells' capacity to maintain genomic stability *in vitro*. This topic will be explored in more detail in the future.

3.3 Validating the origin of CHSS

The species identity of CHSS was determined through amplification and sequencing of the housekeeping gene, cox1. Using BLAST, the cox1 sequences from CHSS were searched and the resulting closest significant sequences with the highest query cover on Genbank were Chinook salmon (accession numbers: JX960927.1, HQ167683.1, AF392054.1 and KX958414.1), providing evidence that CHSS originated from this species (**Table 2**). To our knowledge, the only other available cell line developed from Chinook salmon is the embryonic CHSE-214 (McCain, 1970), or variants of this cell line (Nakano et al, 1993, Collet and Lester, 2011). Through, microsatellite analysis CHSS and CHSE-214 were confirmed to be genetically distinct (**Table 3**). For all 10 of the Chinook salmon microsatellite loci assessed, CHSE-214 and CHSS presented different genotypes. Thus, CHSS is currently the only Chinook salmon cell line developed from both a secondary immune organ, the spleen, and from an individual at a higher developmental age.

For further confirmation of the individual origin of CHSS and CHSE-214, both were genotyped for MH class Iα. Each cell line presented entirely different genotypes for MH class I, but both were homozygous for their respective alleles. Following a BLAST search, the alleles of CHSS and CHSE-214 were observed to have 100% identity to GenBank Chinook salmon MH class I sequences, DQ647923.1 and GU989265.1 respectively. When the individual alleles were aligned (**Figure 4**), there was a surprising difference of 14 nucleotides between the two with a string of 6 nucleotides completely absent in CHSE-214 that were present in CHSS. This would result in a size reduction of 2 amino acids for CHSE-214 when compared to CHSS. Because both alleles have been reported in Chinook salmon populations (Ching et al, 2010, Evans et al, 2010), they appear to be true alleles.

3.4 CHSS can bind dsRNA

To initiate a successful antiviral immune response, cells must first be able to recognize and bind to typical viral PAMPs. When CHSS was exposed to fluorescently labelled pIC, epifluorescence microscopy displayed that the cells were able to successfully bind the dsRNA molecules (**Figure 5**). The staining pattern suggests that dsRNA binding could be plasma membrane-bound, cytosol, and/or endosomal, depending on each examined individual cell. This is the first time this ability has been shown within Chinook salmon cells as CHSE-214 is incapable of dsRNA uptake without transient overexpression of appropriate cell-surface receptors (Jensen et al, 2002b, Monjo et al, 2017). Although there are several examples of other salmonid cell lines capable of binding to extracellular dsRNA (Poynter et al, 2015b), CHSE-214 cannot, highlighting a clear defect in these cells. However, as CHSE-214 was the only Chinook salmon cell line, it was unclear whether the defect was cell line or species specific. As such, CHSS provides an important tool to study functional extracellular dsRNA sensing in Chinook salmon.

3.5 Synthetic dsRNA induces IFN and ISG expression in CHSS

Synthetic dsRNA, or pIC, is a potent inducer of the type I IFN innate immune response (Field et al, 1967). As such, pIC was used to determine the ability of CHSS to produce IFN1 and ISG transcripts in response to extracellular dsRNA. When exposed to varying concentrations of extracellular poly IC, CHSS survival was not influenced by 1, 10 or 100 µg/mL (**Figure 6A**). However, upon exposure to 1 and 10 µg/ml of pIC, CHSS was shown to significantly increase transcript expression of IFN1 at both 3h and 6h post-exposure in a dose-dependent manner (**Figure 6B**). Meanwhile, the ISGs Mx1 and Vig3 also displayed a dose-dependent up-regulation primarily at 6h (**Figure 6C** and **D**). This pIC induced transcript upregulation has been observed in numerous salmonid cell lines (Nygaard et al, 2000, Jensen et al, 2002a, DeWitte-Orr et al, 2007) but interestingly cannot be observed in CHSE-214 without transfection of the synthetic dsRNA (MacDonald and Kennedy, 1979, Jensen et al, 2002b). Because transfection would bypass appropriate receptor binding and uptake, it has been hypothesized that CHSE-214 has a defect somewhere in this mechanism. As CHSS does respond to extracellular dsRNA, the source of the abnormality in CHSE-214 is likely functional in CHSS.

Class A scavenger receptors (SR-A) are integral membrane proteins that mediate dsRNA entry into cells, making them prime candidates for understanding cellular responses to extracellular dsRNA. In mammals, SRAs do not play a role in signaling but rather serve as carrier molecules to deliver extracellular dsRNA to innate antiviral signaling pathways (Nellimaria et al, 2015). SRA receptors can be found on a variety of cell types including those with the epithelial morphology that CHSE-214 and CHSS both present (Limmon et al, 2008, Whelan et al, 2012). To validate whether SR-As were responsible for pIC binding and thus enabling the subsequent type I IFN response observed in CHSS (**Figure 6B-D**), a blocking experiment was performed using known scavenger receptor (SR) competitive and non-competitive ligands. CHSS cells were treated with SR-A competitive or their non-competitive counterpart prior to pIC challenge; after which transcript expression of IFN1 and Vig3 was measured. CHSS cells treated with the SR-A ligand pI followed by pIC were not able to induce IFN or vig-3; however, those treated with the non-competitive ligand, pC, could express IFN1 and Vig3 transcript levels identical to when CHSS was exposed to pIC alone (**Figure 6E** and **F**). These results confirm that CHSS binds and responds to extracellular dsRNA via SR-As. As SR-As are not

3.6 CHSS and CHSE-214 express SCARA3, SCARA4 and SCARA5 transcripts

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As outlined above, CHSS appears to have a fully functional response to extracellular dsRNA, while CHSE-214 has an unknown impairment in dsRNA binding and uptake. Because SR-As have been shown to mediate dsRNA binding in murine and human fibroblasts (DeWitte-Orr et al, 2010), the presence of three SR-A transcripts, SCARA3, SCARA4 and SCARA5, was tested in CHSE-214 and CHSS using primers derived from rainbow trout sequences (Poynter et al, 2018). Interestingly, despite their functional differences, both CHSS and CHSE-214 produced amplicons for all three of the SR-As analyzed. Upon sequencing, the successful amplicons were all found to have very high similarity to the expected sequences in rainbow trout (**Table 4**). These results suggest that, at least at the transcript level, CHSE-214 expresses transcripts for SR-As. This, however, does not mean that these transcripts all produce functional proteins, but the identification of full-length SR-A sequences and the development of Chinook salmon specific antibodies would be required to explore this further. Alternatively, as currently the specific SR-A or SR-As that are responsible for binding dsRNA in fish remain unknown, it is possible that dsRNA may not be a ligand for the three surveyed SR-As. More research is required to fully explore teleostean SR-A function.

A major difference between CHSS and CHSE-214 is the developmental stage of the fish used to create each cell line. As CHSE-214 was established from embryonic Chinook salmon tissue, it is possible that the receptors required to bind and internalize dsRNA or downstream signaling mechanisms were not fully developed at this life stage. When studying the immune response of rainbow trout to live pathogens throughout development, Castro et al (2015) observed that a marked interferon response to VHSV infection was only observed after the first feeding and not during earlier life stages. This could be a protective mechanism for developing organisms as antiviral immune responses can induce cell death to prevent the replication and spread of the pathogen. This trend is not observed in fish models alone as mouse embryonic stem cells (mESCs) also displayed an underdeveloped type I interferon response following exposure to viral pathogens and pIC (Wang et al, 2013). For aquatic organisms, given the high prevalence of viral particles in aquatic systems, it may be that a lack of extracellular dsRNA receptors at earlier life stages will prevent the unnecessary immune system activation and resulting cell death during critical times of development.

4. Conclusions

CHSS is a valuable tool for understanding the cellular physiology and, as described in this study, the immune function of Chinook salmon. The results presented here display that when developed from an adult individual, Chinook salmon cells are capable of binding and subsequently responding to extracellular dsRNA, something that the embryonic CHSE-214 cell line is incapable of. The innate immune response initiated in CHSS appears to be due to SR-A binding as blocking of these receptors abolishes the immune response at the transcript level. Both CHSS and CHSE-214 displayed transcripts for SCARA3, SCARA4 and SCARA5 thus, it appears that further study is required before understanding what is causing the dsRNA binding deficiency in CHSE-214. When it comes to future questions regarding Chinook salmon immunity and cellular function, a cell line established from an adult fish, such as CHSS, provides a vastly improved model system.

Acknowledgements

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References:

1. Banks M.A., Blouin M.S., Baldwin B.A., Rashbrook V.K., Fitzgerald H.A., Blankenship S.M., Hedgecock D., 1999. Isolation and inheritance of novel microsatellites in Chinook salmon (*Oncorhynchus tshawytscha*). The American Genetic Association, 90, 281-288.
2. Bell M.C., 1986. Fisheries handbook of engineering requirements and biological criteria. Fish Passage Development and Evaluation Program. U.S. Army Corps of Engineers, pp 209
3. Brett J.R., Clarke W.C., Shelbourn J.E., 1982. Experiments on thermal requirements for growth and food conversion efficiency of juvenile Chinook salmon *Oncorhynchus tshawytsha.* Canadian Technican Report Aquatic Science No. 1127. DFO, Fisheries Research Branch, Pacific Biological Station, Nanaimo, BC.
4. Ching B., Jamieson S., Heath J.W., Heath D.D., Hubberstey A., 2010. Transcriptional differences between triploid and diploid Chinook salmon (*Oncorhynchus tshawytscha*) during live *Vibrio anguillarum* challenge. Heredity, 104, 224-234.
5. Christensen K.A., Leong J.S., Sakhrani D., Biagi C.A., Minkley D.R., Withler R.E., Rondeau E.B., Koop B.F., Devlin R.H.. 2018. Chinook salmon (*Oncorhynchus tshawytscha*) genome and transcriptome. PLOS One, 13, e0195461.
6. Collet B., Lester K., 2011. Establishment of a Chinook salmon cell line with an inducible gene expression system. In Vitro Cell Dev Biol Anim*,* 47, 695-697.
7. DeWitte-Orr S.J., Collins S.E., Bauer C.M.R., Bowdish D.M., Mossman K.L., 2010. An accessory to the "Trinity": SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. PLOS Pathogens, 6, e1000829.

8. DeWitte-Orr S.J., Leong J.C., Bols N.C., 2007. Induction of antiviral genes, Mx and vig-1, by dsRNA and Chum salmon reovirus in rainbow trout monocyte/macrophage and fibroblast cell lines. Fish and Shellfish Immunology, 23, 670-682.
9. Evans M.L., Neff B.D., Heath D.D., 2010. MHC genetic structure and divergence across populations of Chinook salmon (*Oncorhynchus tshawytscha*). Heredity, 104, 449-459
10. Field A.K., Tytell A.A., Lampson G.P., Hilleman M.R., 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc Natl Acad Sci, 58:1004-1010.
11. Gerstein A.C., McBride R.M., Otto S.P., 2008. Ploidy reduction in *Saccharomyces cerevisiae*. Biology Letters, 4, 91-94
12. Heil F., Hemmi H., Hocherin H., Ampenberger F., Kirschning C., Akira S., Lipford G., Wagner H., Bauer S., 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. Science, 303, 1526-1529.
13. Jacobs, B.L., Langland, J.O., 1996. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology, 219, 339-349.
14. Jensen I., Albuquerque A., Sommer A., Robertsen B., 2002a. Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. Fish and Shellfish Immunology, 13, 311-326
15. Jensen I., Larsen R., Robertsen B., 2002b. An antiviral state induced in Chinook salmon embryo cells (CHSE-214) by transfection with the double-stranded RNA poly I:C. Fish and Shellfish Immunology, 13, 367-378.
16. Johnson B.M., Kemp B.M., Thorgaard G.H., 2017. Increased mitochondrial DNA diversity in ancient Columbia River basin Chinook salmon *Oncorhynchus tshawytscha*. PLoS One, 13, e0190059.
17. Kavaliauskis A., Arnemo M., Speth M., Lagos L., Rishovd A.L., Estepa A., Griffiths G., Gjoen T., 2016. Protective effect of recombinant VHSV-G vaccine using poly(I:C) loaded nanoparticles as an adjuvant in zebrafish (*Danio rerio*) infection model. *Developmental and Comparative Immunology,* 61:248-257.
18. Knudsen C.M., Schroder S.L., Busack C.A., Johnston M.V., Pearsons T.N., Bosch W.J., Fast D.E., 2006. Comparison of life history traits between first-generation hatchery and wild upper Yakima River spring Chinook salmon. Transactions of the American Fisheries Society, 135, 1130-1144.
19. Kuzman I.V., Schwarz T.M., Ilinykh P.A., Jordan I., Ksiazek T.G., Sachidanandam R., Basler C.F., Bukreyev A., 2017. Innate immune responses of bat and human cells to filoviruses: commonalities and distinctions. Journal of Virology, 91, e02471-16.

20. Lehnert S.J., Pitcher T.E., Devlin R.H., Heath D.D., 2016. Red and white Chinook salmon: genetic divergence and mate choice. Molecular Ecology, 25, 1259-1274.
21. Li X., Ma C., Qin Y., Li Y., Wu D., Bai L., Pei A., 2015. Establishment and characterization of fin cell lines from diploid, triploid and tetraploid oriental weatherfish (*Misgurnus anguillicaudatus*). Fish Physiology and Biochemistry, 41, 661-672.
22. Limmon G.V., Arredouani M., McCann K.L., Minor R.A.C., Kobzik L., Imani F., 2008. Scavenger receptor class-A is a novel cell surface receptor for double-stranded RNA. FASEB J, 22, 159-167.
23. MacDonald R.D., Kennedy J.C., 1979. Infectious Pancreatic Necrosis Virus persistently infects Chinook salmon embryo cells independent of interferon. Virology, 95, 260-264.
24. Marine K.R., 1997. Effects of elevated water temperature on some aspects of the physiological and ecological performance of juvenile chinook salmon (*Oncorhynchus tshawytsha*): Implications for management of California's Central Valley salmon stocks. M.S. thesis. University of California, Davis.
25. McCain B.B., 1970. The Oregon Sockeye Salmon Virus: A. Biophysical Biochemical Characteristics B. Antigenic Relationship to Two Other Salmonid Viruses. Doctoral Thesis, Oregon State University, Oregon.
26. Monjo A.L., Poynter S.J., DeWitte-Orr S.J., 2017. CHSE-214: A model for studying extracellular dsRNA sensing *in vitro*. Fish and Shellfish Immunology, 68, 266-271.
27. Nakano H., Wada Y., Hasobe M., 1993. Chinook salmon embryo (CHSE-sp) cell for fish virus research. In Vitro Cellular and Developmental Biology, 29, 265-267.
28. Nellimarla S., Baid K., Loo Y., Gale M.J., Bowdish D.M., Mossman K.L., 2015. Class A scavenger receptor-mediated dsRNA internalization is independent of innate antiviral signaling and does not require P13K activity. *The Journal of Immunology,* 185:3858-3865.
29. Nuti R.V., Giorgetti L., Tonelli M., Martini G., 1992. Ploidy reduction and genome segregation in cultured carrot cell lines. I. Prophase chromosome reduction. Plant Cell, Tissue and Organ Culture, 30, 107-114.
30. Nygaard R., Husgard S., Sommer S., Leong J.C., Robertsen B., 2000. Induction of Mx protein by interferon and double-stranded RNA in salmonid cells. Fish and Shellfish Immunology, 10, 435-450.
31. O'Connell M., Danzmann R.G., Cornuet J., Wright J.M., Ferguson M.M., 1997. Differentiation of rainbow trout (*Oncorhynchus mykiss*) populations in Lake Ontario and the evaluation of the stepwise mutation and infinite allele mutation models using microsatellite variability. Canadian Journal of Fisheries and Aquatic Sciences, 54, 1391-1399.

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32. Ohlberger J., Ward E.J., Schindler D.E., Lewis B., 2018. Demographic changes in Chinook salmon across the Northeast Pacific Ocean. Fish and Fisheries, 19, 533-546.
33. Oidtmann B., Dixon P., Way K., Joiner C., Bayley A.E., 2017. Risk of waterborne virus spread – a review of survival of relevant fish and crustacean viruses in the aquatic environment and implications for control measures. Reviews in Aquaculture*,* 0, 1-29
34. Olsen J.B., Bentzen P., Seeb J.E., 1998. Characterization of seven microsatellite loci derived from pink salmon. Molecular Ecology, 7, 1087-1089.
35. O'Reilly P.T., Hamilton L.C., McConnell S.K., Wright J.M., 1996. Rapid analysis of genetic variation in Atlantic salmon (*Salmo salar*) by PCR multiplexing of dinucleotide and tetranucleotide microsatellites. Canadian Journal of Fish Aquatic Sciences, 53, 2292-2298
36. Poynter, S.J., DeWitte-Orr, S.J., 2016. Fish interferon-stimulated genes: The antiviral effectors. Developmental & Comparative Immunology, 65, 218-225.
37. Poynter S.J., DeWitte-Orr S.J., 2017. Visualizing virus-derived dsRNA using antibody-independent and -dependent methods. *Methods in Molecular Biology,* 1656:103-118.
38. Poynter S., Lisser G., Monjo A., DeWitte-Orr S., 2015a. Sensors of infection: viral nucleic acid PRRs in fish. Biology, 4, 460-493.
39. Poynter S.J., Monjo A.L., DeWitte-Orr S.J., 2018. Identification of three class A scavenger receptors from rainbow trout (*Oncorhynchus mykiss*): SCARA3, SCARA4, and SCARA5. Fish and Shellfish Immunology, 76, 121-125.
40. Poynter S., Weleff J., Soares A.B., DeWitte-Orr S., 2015b. Class-A scavenger receptor function and expression in the rainbow trout (*Oncorhynchus mykiss*) epithelial cell lines RTgutCC and RTgill-W1. Fish and Shellfish Immunology, 44, 138-146
41. Rexroad III C.E., Coleman R.L., Martin A.M., Hershberger W.K., Killefer J., 2001. Thirty-five polymorphic microsatellite markers for rainbow trout (*Oncorhynchus mykiss*). Animal Genetics, 32, 317-319.
42. Richter A., Kolmes S.A., 2005. Maximum temperature thermal limits for Chinook, coho, and chum salmon, and steelhead trout in the Pacific Northwest. Reviews in Fisheries Science, 13, 23-49.
43. Rieger A.M., Barreda D.R., 2016. Accurate Assessment of Cell Death by Imaging Flow Cytometry. In: Barteneva N., Vorobjev I. (eds) Imaging Flow Cytometry. Methods in Molecular Biology, vol 1389. Humana Press, New York, NY

44. Scribner K.T., Gust J.R., Fields R.L., 1996. Isolation and characterization of novel salmon microsatellite loci: cross-species amplification and population genetic applications. Canadian Journal of Fish Aquatic Sciences, 53, 833-841.

45. Semple S.L., Kellendonk C.J., Al-Hussinee L., MacInnes J.I., Lumsden J.S., Dixon B., 2018. Serum IgM, MH class IIβ genotype and respiratory burst activity do not differ between rainbow trout families displaying resistance or susceptibility to the coldwater pathogen, *Flavobacterium psychrophilum*. Aquaculture, 483, 131-140

46. Strandskog G., Villoing S., Iliev D.B., Thim H.L., Christie K.E., Jorgensen J.B., 2011. Formulations combining CpG containing oliogonucleotides and poly I:C enhance the magnitude of immune responses and protection against pancreas disease in Atlantic salmon. *Developmental and Comparative Immunology,* 35:1116-1127.

47. Suttle C.A., 2005. Viruses in the sea. Nature, 437:356-361.

48. Thim H.L., Villoing S., McLoughlin M., Christie K.E., Grove S., Frost P., Jorgensen J.B., 2014. Vaccine adjuvants in fish vaccines make a difference: comparing three adjuvants (Montanide ISA763A oil, CpG/Poly I:C combo and VHSV glycoprotein) alone or in combination formulated with an inactivated whole salmonid alphavirus antigen. *Vaccines,* 2:228-251.

49. Williamson K.S., Cordes J.F., May B., 2002. Characterization of microsatellite loci in chinook salmon (*Oncorhynchus tshawytscha*) and cross-species amplification in other salmonids. Molecular Ecology Notes, 2, 17-19.

50. Vcelar S., Jadhav V., Melcher M., Auer N., Hrdina A., Sagmeister R., Heffner K., Puklowski A., Betenbaugh M., Wenger T., Leisch F., Baumann M., Borth N., 2018. Karyotype variation of CHS host cell lines over time in culture characterized by chromosome couting and chromosome painting. Biotechnology and Bioengineering, 115, 165-173.

51. Vo N.T.K., Bender A.W., Ammendolia D.A., Lumsden J.S., Dixon B., Bols N.C., 2015a. Development of a walleye spleen stromal cell line sensitive to viral hemorrhagic septicemia virus (VHSV IVb) and to protection by synthetic dsRNA. Fish and Shellfish Immunology, 45, 83-93.

52. Vo N.T.K., Bender A.W., Lee L.E.J., Lumsden J.S., Lorenzen N., Dixon B., Bols N.C., 2015b. Development of a walleye cell line and use to study the effects of temperature on infection by viral hemorrhagic septicaemia virus (VHSV) group IVb. Journal of Fish Diseases, 38, 121-136.

53. Vo N.T.K., Bender A.W., Lumsden J.S., Dixon B., Bols N.C., 2016. Differential viral haemorrhagic septicemia virus genotype IVb infection in fibroblast and epithelial cell lines from walleye caudal fin at cold temperatures. Journal of Fish Diseases, 39, 175-188.

54. Wang R., Wang J., Paul A.M., Acharya D., Bai F., Huang F., Guo Y.L., 2013. Mouse embryonic stem cells are deficient in type I interferon expression in response to viral infections and double-stranded RNA. Journal of Biological Chemistry, 288, 15926-15936.

55. Whelan F.J., Meehan C.J., Golding G.B., McConkey B.J., Bowdish D.M.E., 2012. The evolution of the class A scavenger receptors. BMC Evolutionary Biology, 12, 227.

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Table 1: Primer sequences used to amplify Chinook salmon genes

Gene	Sequence (5`-3`)	Annealing Temp	Accession No. or Reference
Cox1	F- CCTCAGTTGATCTGACGA R- CACGAGTGTCAACGTCTA	52°C	*JX960927.1*
B-Actin	F- GTCACCAACTGGGACGACAT R- GTACATGGCAGGGGGTGTTGA	55°C	Monjo et al, 2017
IFN1	F- AAAACTGTTTGATGGGAATATGAAA R- CGTTTCAGTCTCCTCTCAGGTT	55°C	Monjo et al, 2017
Mx1	F- CGGAGTTCGTCTCAACGTCT R- CCCTTCCACGGTACGTCTTC	55°C	Monjo et al, 2017
Vig3	F- ACCCAGTTCAAAGCCAAGGT R- CCCTCGTGAATCAGCCTCTG	55°C	Monjo et al, 2017
SCARA3	F- GTTGAGCGGTCTGAAGTCCA R- TCAAGACACCGTGTCTGGGA	55°C	*MF664681.1*
SCARA4	F- TCCCTGGGAGAGATGGACAG R- CTAATGATGCTCATCATACCGC	60°C	*MF664680.1*
SCARA5	F- CCGTGTATCCGTCCTGAACC R- TGTACCGTGCATCATGGCTT	55°C	*AQW38823.1*

Table 2: Validating species identity of CHSS through Cox1 Barcoding

Gene	Length (bp)	% Identity to Chinook salmon Sequences
Cytochrome C Oxidase Subunit 1 (Cox1)	492	100%, *JX960927.1* 100%, HQ167683.1 100%, AF392054.1 99%, KX958414.1

Table 3: Microsatellite genotypes of CHSS and CHSE-214

Microsatellite Loci	CHSS	CHSE-214
Ogo4	170/185	165/177
Oneu8	196/196	183/188
Ots1	207/207	207/217
OtsG432	138/138	134/134
OtsG474	196/200	212/212
Omm1135	223/223	243/243
Omy325	134/134	108/121
Oneu14	241/241	167/253

Ots4	173/173	179/185
Ssa85	177/197	147/183

Table 4: Presence of SR-A transcripts in CHSS and CHSE-214

SR-A	CHSE-214	CHSS	Length (bp)	% similarity to rainbow trout
SCARA3	✓	✓	777	98.3% *MF664681.1*
SCARA4	✓	✓	661	98.6% *MF664680.1*
SCARA5	✓	✓	224	99.6% *AQW38823.1*

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Figure Legends:

Figure 1: Development of the Chinook salmon spleen cell line, CHSS. (A) Initial 3-month tissue explant outgrowth from the Chinook salmon spleen. Preliminary cells had a fibroblastic-like morphology. (B) After one year of growth, cells appeared more epithelial-like and were prone to growing on top of each other resulting in the slow development of a monolayer. (C) In earlier passages (passage 2), two distinct morphologies could be observed in what would eventual become CHSS. (D) Upon subsequent passages (passage 15), the epithelial-like morphology became predominant. Magnification 100x

Figure 2: Optimal culture conditions for the growth of CHSS. (A) Effect of temperature on growth. CHSS cells were plated at 200,000 cells/well in 20% FBS supplemented media, incubated at 4, 14, 18 or 26^{o}C and counted on days 3, 7, 10 and 14. (B) Influence of FBS concentration on CHSS proliferation. Cells were plated at 200,000 cells/well. Following overnight adherence, triplicate wells received media supplemented with either 5%, 10%, 15% or 20% FBS and were then counted on days 3, 7, 10 and 14. For both experiments, percent growth was calculated with respect to the average day 0 cell count. All cell counts were done in triplicate and are represented as means ±SD.

Figure 3: Ploidy analysis of Chinook salmon cells. After permeabilizing the cells with 70% ethanol and treatment with RNase A, both cell types were exposed to 1 mg/mL of propidium iodide before assessing the DNA content of CHSE-214 (A) and CHSS (B) individually using flow cytometry. The data represents the results of three independent experiments.

Figure 4: Sequence alignment of CHSS and CHSE-214 MH class I (MHI) α1 alleles. To limit PCR artefacts, MHI sequences for both cell cultures were determined using two individual PCR reactions with 25 clones from each PCR reaction sequenced. Only those alleles that were observed in both PCR reactions were considered true MHI alleles. The primers used amplify the peptide binding groove of the MHI α1 domain.

Figure 5: CHSS is capable of binding to extracellular dsRNA. (A) Negative control: cells received only media. (B) CHSS cells following a 1h incubation with 50 µg/mL of fluorescently labelled dsRNA (green). Magnification 400x.

Figure 6: The impact of pIC on CHSS viability and transcript expression of IFN and ISGs. (A) An Alamar Blue assay was used to determine the survival of CHSS following 24h and 48h exposure to pIC at concentrations of 0, 1, 10 and 100 µg/mL. (B-D) CHSS was treated with pIC at concentrations of 0, 1 or 10 µg/mL and cells were collected at 3h and 6h post-exposure. Following RNA extraction and cDNA synthesis, transcript levels of IFN1 (B) and two ISG transcripts, Mx1 (C) and Vig3 (D) were measured via qRT-PCR. (E-F) After 30 minutes of pre-treatment with 250 µg/mL of the SR-A competitive and non-competitive ligands, pI and pC respectively, CHSS was exposed to 1 µg/mL of pIC for 3h and cells were collected. Following RNA extraction and cDNA synthesis, transcript levels of IFN1 (E) and Vig3 (F) were measured using qRT-PCR. All panels represent three independent experiments and are presented as means ±SEM.

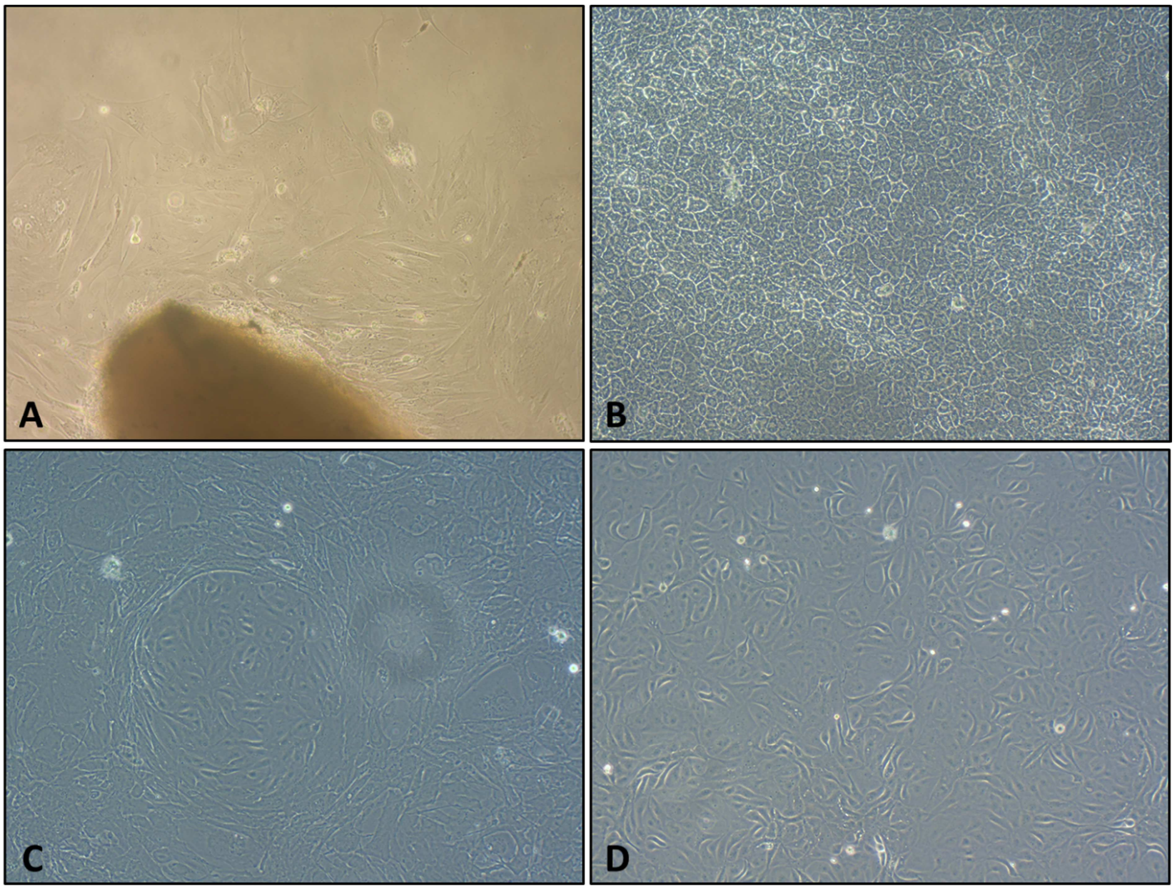

Figure 1

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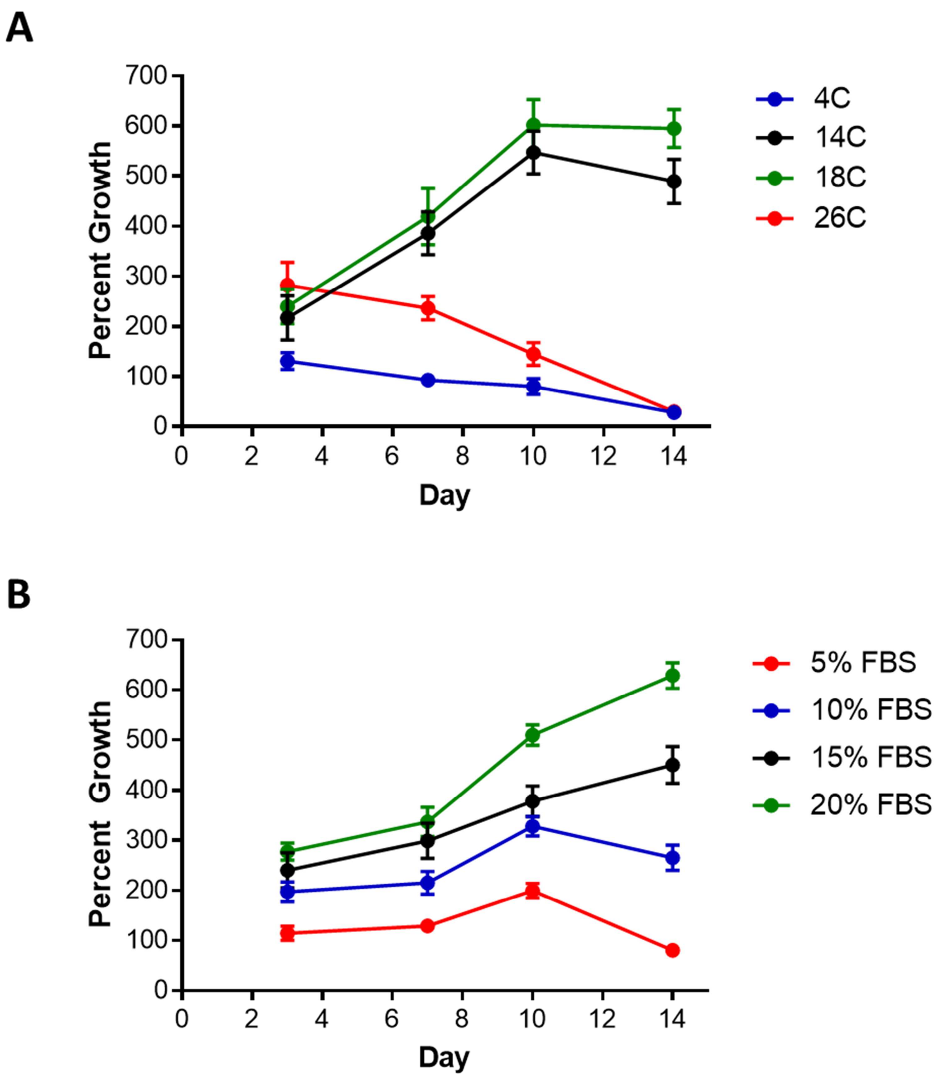

Figure 2

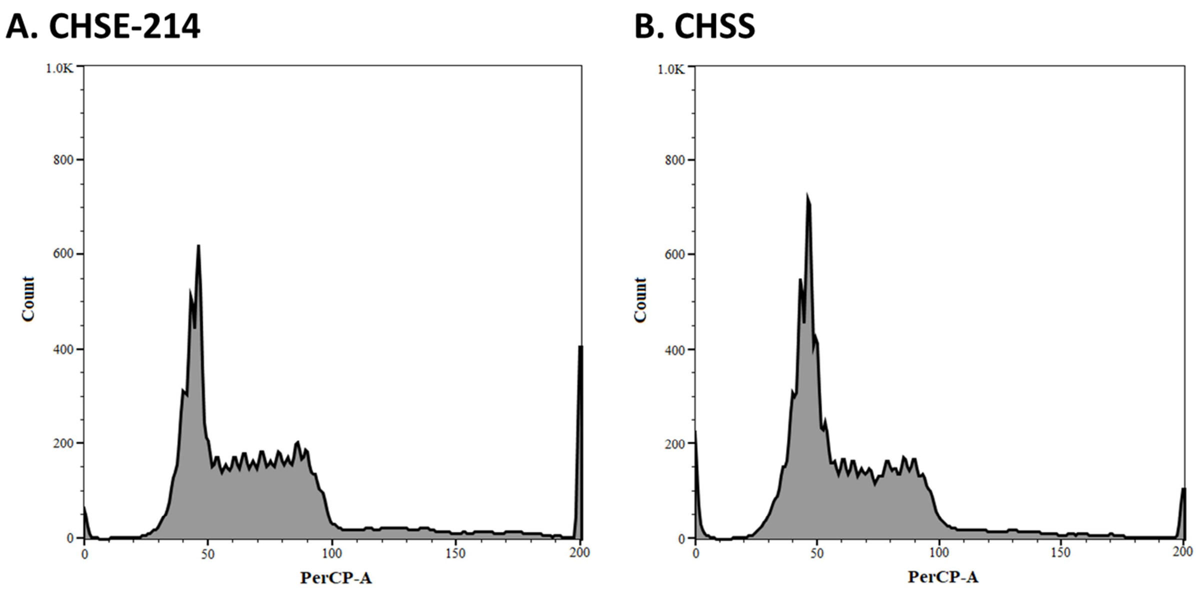
A. CHSE-214
B. CHSS
1.0K
800
600
400
200
0
Count
0
50
100
150
200
PerCP-A

Figure 3

```
CHSE-214        TTCTTCACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGATTGTGGGGATGGTG  60
CHSS            TTCTTCACCGCATCTTCTGAAGTTCCCAACTTCCCAGAGTTCGTGGTTGTGGGGATGGTG  60
                ********************************************* **************

CHSE-214        GATGGTGTTCAGATGGTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC 120
CHSS            GATGGTGTTCAGATGTTTCACTATGACAGCAACAGCCAGAGAGCGGTGCCCAAACAGGAC 120
                *************** ********************************************

CHSE-214        TGGGTAAACAAGGCAGCAGAC------CCACAGTACTGGGAGAGGAACACTGGGAATGGC 174
CHSS            TGGATGAACAAGGCAGCAGAAACACTGCCACAGTACTGGGAGAGGAACACTGGGAATTGC 180
                *** * **************       ****************************** **

CHSE-214        AAGGGTGCCCAGCAGACTTTCAAAGCCAACATCGATATTGCAAAGCAG             222
CHSS            AAGGGTGACCAGCAGACTTTCAAAGCCAACATCGATATTGTAAAGCAG             228
                ******* ******************************** *******
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Figure 4

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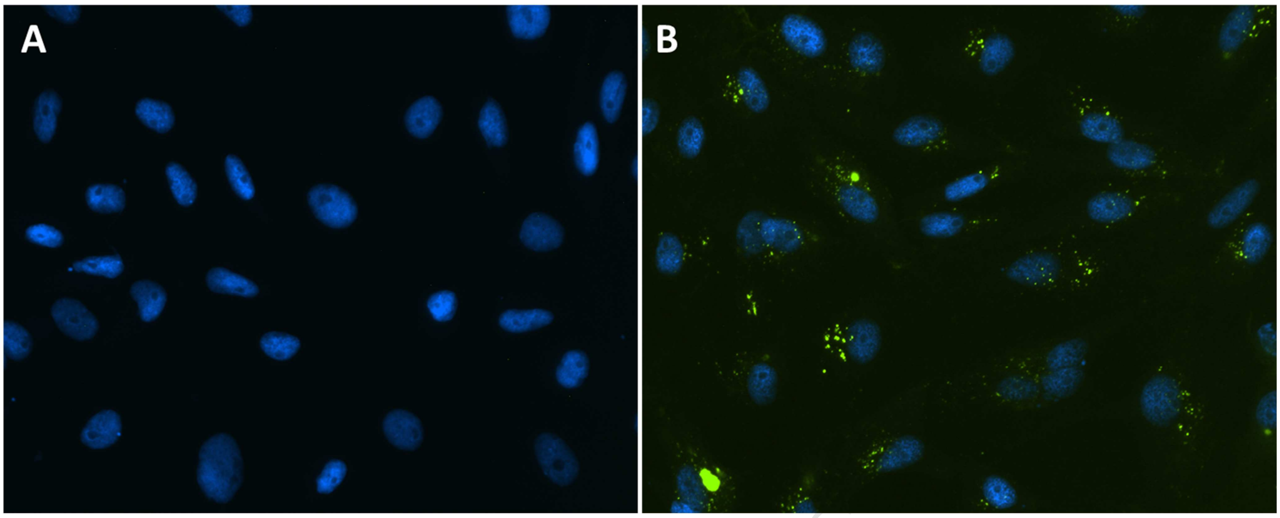

Figure 5

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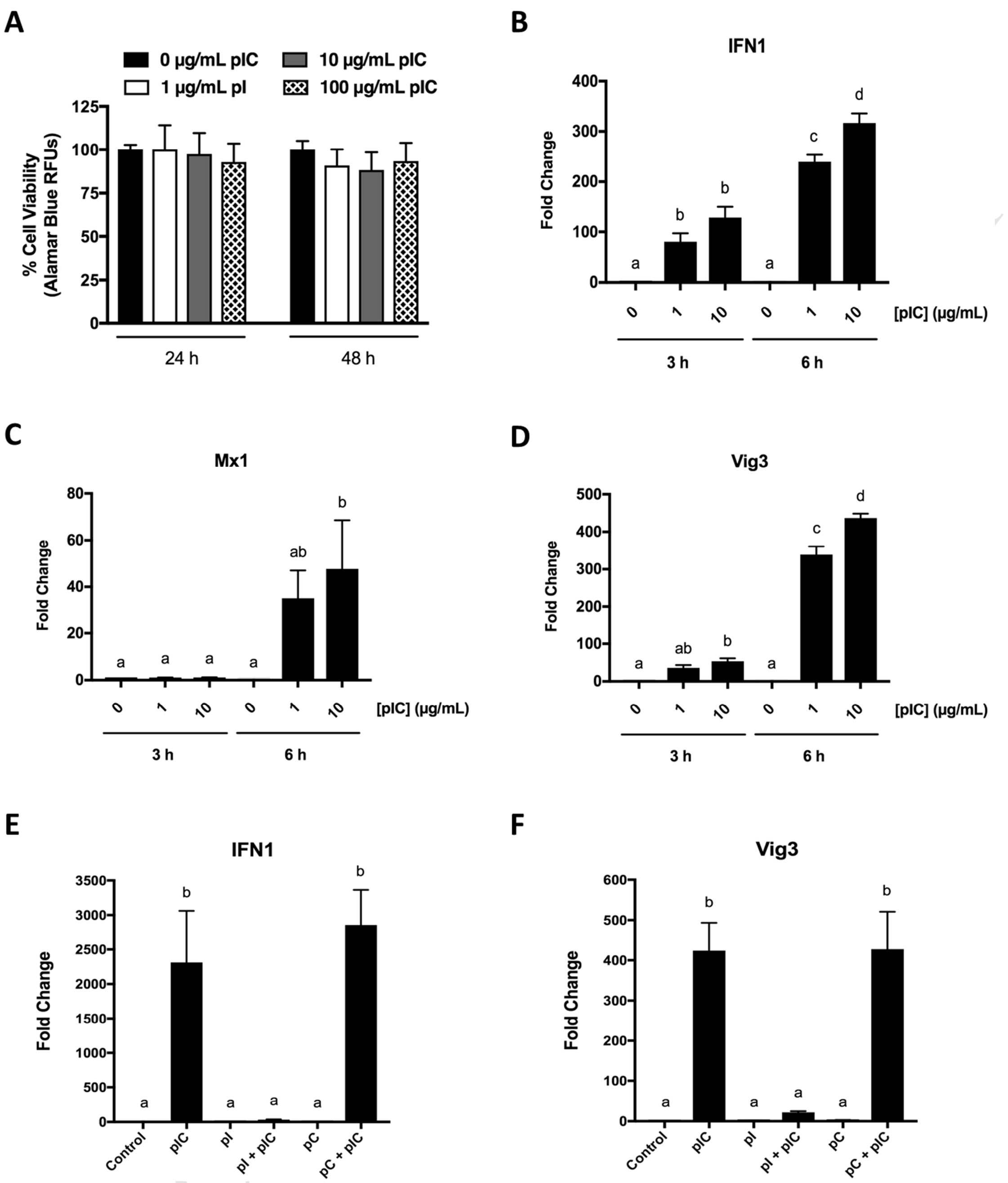
A
0 µg/mL pIC
10 µg/mL pIC
1 µg/mL pI
100 µg/mL pIC
% Cell Viability (Alamar Blue RFUs)
125
100
75
50
25
0
24 h
48 h
B
IFN1
Fold Change
400
300
200
100
0
a
b
b
a
c
d
0
1
10
[pIC] (µg/mL)
3 h
6 h
C
Mx1
Fold Change
80
60
40
20
0
a
ab
b
[pIC] (µg/mL)
3 h
6 h
D
Vig3
Fold Change
500
400
300
200
100
0
a
ab
b
c
d
[pIC] (µg/mL)
3 h
6 h
E
IFN1
Fold Change
3500
3000
2500
2000
1500
1000
500
0
a
b
Control
pIC
pI
pI + pIC
pC
pC + pIC
F
Vig3
Fold Change
600
500
400
300
200
100
0
a
b
Control
pIC
pI
pI + pIC
pC
pC + pIC

Figure 6

Highlights:

1. Development of a novel Chinook salmon cell line, CHSS
2. CHSS can bind extracellular dsRNA, unlike CHSE-214
3. Both CHSE-214 and CHSS express scavenger receptors
4. CHSS can be used to study Chinook salmon antiviral responses *in vitro*

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