Final Report

Aquafin CRC

Aquafin CRC – FRDC Southern Bluefin Tuna Aquaculture Subprogram: tuna cell line development and their application to tuna aquaculture health surveillance

Mark St. J. Crane and Lynette M. Williams

August 2005

Aquafin CRC Project 3.2
(FRDC Project No. 2001/200)

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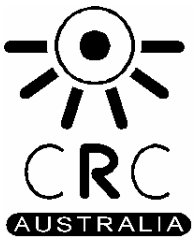

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1.0. NON-TECHNICAL SUMMARY

Aquafin CRC Project 3.2
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Aquafin CRC – FRDC Southern Bluefin Tuna Aquaculture Subprogram: tuna cell line development and their application to tuna aquaculture health surveillance

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OUTCOMES ACHIEVED

The aim of the project was to develop continuous cell lines, through the use of mutagenesis of cultured cells derived from Southern bluefin tuna (SBT) tissues, to improve our capacity to isolate and characterise viruses and further develop the health surveillance program for SBT aquaculture sector. Thus the cell lines would be susceptible to infection by a range of viruses, such that diagnostic procedures using these cell lines could be developed. In addition, the availability of these cell lines would facilitate the development of other diagnostic procedures (immunoassays and molecular tests) for viral diseases of SBT.

A prerequisite for the development of continuous (immortal) cell lines is the establishment of primary (mortal) cell cultures that have a limited life-span but are capable of cellular replication. During this period of limited cellular division the primary cell cultures can be manipulated to encourage production of cells with the potential for unlimited cellular replication.

In other systems such as those developed for mammalian cells, nutrient media, supplemented with factors which stimulate growth, have been established for decades. Previous studies have demonstrated that these media can be used to establish cell lines from other marine fish species. This project, drawing on previous experience from these systems, was aimed at the development of continuous SBT cell lines.

Preliminary studies were undertaken to reproduce the establishment of primary cell cultures from SBT using the standard cell culture media available commercially. While the initial aim of establishing primary cultures was achieved it became evident that such cultures had a very restricted potential for cellular division. It was concluded that the reason for this was due to the age of the fish used as the source of tissue for establishing the primary cultures. In most other systems fish that were less than 1 year-old or less than 1 kg in weight were used as the starting material.

Due to the lack of high levels of cellular replication, the use of transforming agents was unlikely to be an effective approach. Nevertheless, cultures were nurtured to encourage transformation. However, despite several attempts, all cultures senesced over a period of a few weeks/months, did not transform and eventually died.

Thus while the main objective was not achieved, results from this project have demonstrated unequivocally that current materials (e.g. existing culture media) and methods (tissue explants and enzymatic dissociation) are adequate for the establishment of primary cell cultures. It is likely that younger fish will be required as starting material in order to develop immortal, continuous SBT cell lines.

KEYWORDS: SBT primary cell culture, cell lines, viruses

2.0. ACKNOWLEDGEMENTS

The authors acknowledge the contributions of Tuckweng Kok and Lyn Payne (Virology Laboratory, Institute for Medical and Veterinary Science, Adelaide SA), David Ellis (Fishing Industry Services), Jeff Buchanan (SARDI), Craig Hayward (U. Tasmania) and the management and staff of Sekol Farmed Tuna, Kailis Pty Ltd (Tuna), AJKA Pty Ltd, Blaslov Fishing Company and the Stehr Group to this project. The efforts of Graeme Dyke and World-link International to ensure the prompt air-freight of tuna samples to AAHL are also appreciated. In addition, the authors thank the Aquafin Cooperative Research Centre (CRC), the Fisheries Research and Development Corporation (FRDC), the SBT Aquaculture Subprogram (in particular Steven Clarke) and the Aquafin CRC Health Subprogram (in particular Barbara Nowak) for their support.

3.0. BACKGROUND

Southern bluefin tuna (SBT) aquaculture involves the ranching of sea-caught tuna in marine net-pens. Fish, 2-5 years old, are caught from the Great Australian Bight wild-tuna-fishing grounds and transferred from tow-cages to grow-out cages at Port Lincoln, South Australia. SBT are fed for 3-8 months, and then harvested. All sea cages are emptied within 3-9 months of the initial stocking. A major limitation on the industry is that the number of wild fish caught is restricted by an international quota system. It is recognised that this limitation could be circumvented if the life-cycle of the SBT could be closed.

A major development expected in the tuna aquaculture sector over the next decade or so is the closing of the SBT, *Thunnus maccoyii*, life-cycle. In order to capitalise on this advancement it will be essential that healthy broodstock can be identified so that disease-free eggs and fry can be produced. For other farmed finfish, it is these early life-cycle stages which tend to be most susceptible to infectious diseases including those caused by bacterial and viral pathogens (Woo & Bruno, 1999). It would be highly desirable that procedures for the isolation and identification of bacterial and viral pathogens of tuna should be established in anticipation of the requirement to test potential broodstock, eggs, fry etc. for the presence of potential pathogens.

To date, procedures for the isolation and identification of bacterial and viral pathogens of SBT have not been developed probably due to the fact that the SBT life-cycle has not been closed and little is known about SBT pathogens (Munday et al., 1997; Nowak et al., 2003; Colorni, 2004). Wild-caught SBT are usually 2-5 years old and would represent survivors of any infectious disease that may have occurred at an earlier time in their life. Indeed these “survivors” may be immune to pathogens to which they had been exposed previously, but may still carry low levels of infectious agents. These clinically normal “carriers” may act as reservoirs of infectious agents which could be passed on to other, non-immune and susceptible individuals including eggs and fry, raised in intensive culture conditions.

In order to develop further the health surveillance program for the SBT aquaculture industry, specific tools are required. For the isolation and identification of viral pathogens of any animal host species the availability of continuous cell lines susceptible to the relevant viruses provides one of these basic tools. Currently, and to the authors’ knowledge, there are no continuous tuna cell lines. This project is aimed at the development of such cell lines and their utilisation in an enhanced health surveillance program for the SBT aquaculture industry.

In addition, the availability of continuous cell lines will improve responsiveness to viral disease outbreaks, should they occur, following closure of the SBT life-cycle. The incorporation of the use of such cell lines into any propagation project would provide an enhanced capability for both the NBT and the YFT projects occurring overseas. The use of these cell lines would improve our ability to identify healthy tuna broodstock, eggs and fry. It is anticipated that through the use of tuna cell lines, virus-free stock could be selected and thus

reduce the increased costs associated with rearing infected fish. Fish health specialists would be more capable of monitoring tuna health leading to increased productivity due to the use of virus-free stock.

4.0. NEED

The use of fish cell lines, both as a research tool and a diagnostic tool, has played a major role in the development of salmonid and cyprinid aquaculture worldwide (Wolf & Mann, 1980). The commercial success of these finfish aquaculture industries is due, in part, to the development of fish cell lines which are used to monitor farmed fish populations for the presence of specific viral pathogens (OIE, 2003). Based on the results of such health surveillance programs disease-free stocks can be kept isolated from infected stock through restrictions in fish movements. The current lack of continuous tuna cell lines suitable for the isolation and growth of viral pathogens of tuna could be a serious obstacle to effective disease control in tuna hatcheries and nurseries which, in turn, could have a significant negative impact on the future development of the tuna aquaculture sector. It is noteworthy that viral infections of a tuna species (*Thunnus thynnus*) have been documented (Matsuoka et al., 1996). Moreover, other viral pathogens such as marine nodaviruses (Nishizawa et al., 1997) and birnaviruses (Reno, 1999) tend to have a broad host range and should be considered a significant risk. In deed, it is generally accepted that many of the diseases of aquaculture species originate in wild, marine species (Dixon, 1999; Munday et al., 2002).

Development of diagnostic tools for identification of viral pathogens in other systems has been reliant on the availability of continuous cell lines for virus cultivation (Murray et al. 1995). Isolation and growth of viral pathogens in susceptible cell lines provide an almost limitless supply of partially purified virus for the development of improved diagnostic procedures for these pathogens (Crane & Bernoth, 1996). In order to be able to develop similar systems to service the farmed tuna sector, there is a need for continuous tuna cell lines.

The aim of this project is to develop continuous SBT cell lines to improve our capacity to isolate and characterise tuna viruses, and to enhance our response to new pathogens that may threaten farm production. Identification of disease-free broodstock, eggs and fry is desirable for the further development of the SBT aquaculture sector.

5.0. OBJECTIVES

5.1. Establish primary cell cultures from southern bluefin tuna (SBT) tissues, larvae or fry.

5.2. Select sub-populations of cell cultures which display continuous cell division.

5.3. Clone cells which display features of immortal cell lines.

5.4. Characterise the major features of the tuna cell lines important for their application as a diagnostic tool.

5.5. Determine susceptibility of cell lines to viral pathogens of marine finfish.

6.0. METHODS

6.1. Literature Review

Literature searches were undertaken, accessing fisheries and fish health databases, to establish whether there were any immortal (continuous) tuna cell lines reported in the literature. In addition, where there were reports on the development of any marine fish cell lines, the methods used for their development were noted and incorporated into the methods used here.

6.2. Access to Southern Bluefin Tuna

For the generation of immortal cell lines, it is recommended that the source fish (from which the tissues are to be obtained) should be as young as possible (Nicholson, 1985). Using young fish would increase the likelihood of obtaining primary and secondary cultures with vigorously replicating cells. When this project was initially conceived, it was anticipated that young Southern Bluefin Tuna, SBT (less than one-year old or less than 1 kilogram in weight) would be available from the (subsequently discontinued) Propagation Project. Following commencement of this project, it became evident that this requirement would not be met but it was deemed that the SBT cell line project should continue due to the potential benefit immortal SBT cell lines would confer to the industry. When obtaining SBT for generation of cell cultures, the aim was to target the youngest fish available at the time. Access to SBT, as a source of tissues, was coordinated by Aquafin CRC staff at Port Lincoln, South Australia. SBT were obtained from either the research farm site or purchased from SBT farmers.

During the course of the project, the use of other fish species (yellowfin tuna, yellowtail kingfish or northern bluefin tuna) was considered and discounted. Reasons for not using these other species included the lack of availability in Australia and/or lack of support by industry. While other tuna species are available overseas as well as young fish, availability of appropriate facilities, equipment and reagents was a significant issue. Furthermore, it was considered unlikely that primary cell cultures established overseas could be subsequently import into Australia for general use, nor would it be possible to import live fish or tissues to establish primary cultures for general use in Australia.

6.3. Collection of Fish Tissues

It is well-established that fish tissues, once collected, tend to autolyse rapidly. Therefore, for initial experiments, tuna tissues were obtained aseptically (see figure 1) from farmed SBT at Port Lincoln and transported, on ice, to a local laboratory for processing. The virology laboratory, Institute for Medical and Veterinary Science (IMVS), Adelaide, SA agreed to collaborate on the early phases of this project. The cell culture facilities at IMVS virology laboratory were made available for initial tissue processing and establishment of primary cultures from the first few SBT that were available.

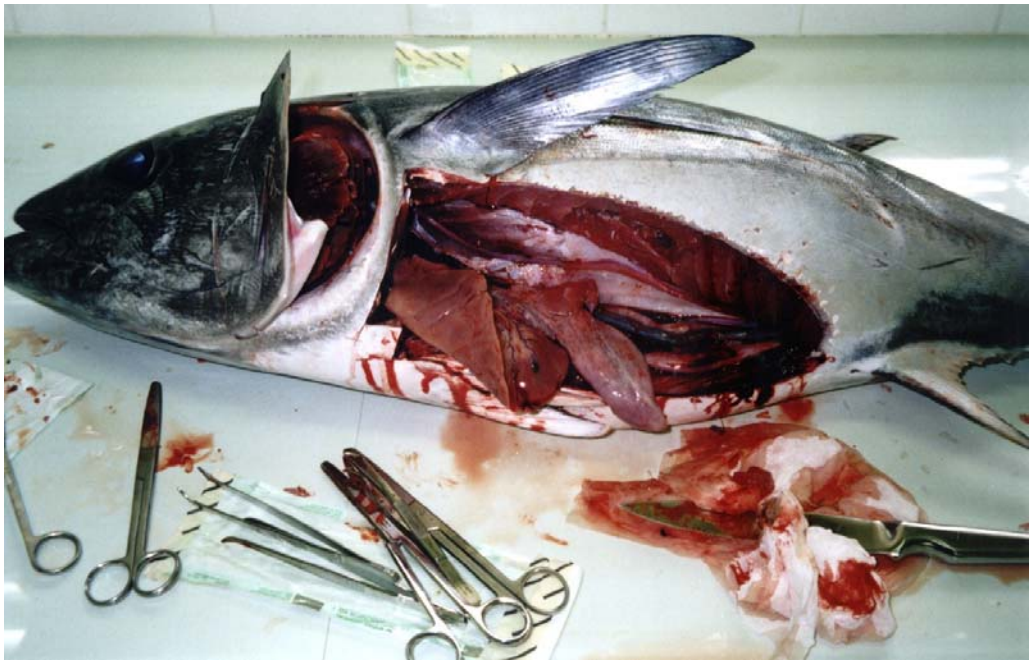

Figure 1. Dissection of SBT to obtain tissues for cell culture.

Subsequently, it was demonstrated that tissues could be obtained by local CRC staff and transported, on ice, directly to the Australian Animal Health Laboratory (AAHL), Geelong where they could be processed promptly with primary cultures established. Thus it was demonstrated that a local laboratory was not required and IMVS was not utilised after the first year of the project.

For each experiment, tissues were sampled from a one or two SBT (approximately 10-20 kg in weight). Initially, the tuna were dissected aseptically and the following tissues collected into sterile transport medium for transfer, on ice, to IMVS, Adelaide or AAHL, Geelong:

- Anterior kidney
- Posterior kidney
- Spleen
- Heart
- Liver
- Muscle
- Gonad
- Abraded fin
- Tumour

Medium used for transport of excised tissue from the field was Eagle's minimum essential medium with Earle's salts (EMEM) (Gibco-BRL Cat # 41500-034) supplemented with 10mM hepes (BDH Cat # 44285 6A) buffer, 2mM glutamine (ICN Biomedicals Inc. 1580115), 2% (v/v) foetal bovine serum (CSL) 50µg/ml gentamycin sulphate (Sigma Cat # G-3623) and 5µg/ml amphotericin B (Bristol-Myers Squibb Co "Apothecon" Fungizone-Amphotercin B).

6.4. Tissue Processing

At the laboratory (either IMVS or AAHL) each tissue was processed according to standard procedures for establishment of primary cell cultures (Freshney, 1994; Nicholson, 1985). Tissues were rinsed in phosphate buffered saline, PBSA pH 7.4, (without Ca^{2+} and Mg^{2+} ions), supplemented with 50 µg/ml gentamycin sulphate and 5µg/ml amphotericin B, to remove blood. The tissues were diced and rinsed further to remove blood. Spleen was particularly bloody, required extensive rinsing and, even then, not all red blood cells were removed.

When the washing solution showed no further red colour, the diced tissues were then prepared for culture. Methods used included: (i) Explant; (ii) Tissue dissociation by enzymatic digestion, using trypsin, trypsin/EDTA, dispase and/or collagenases; (iii) Tissue disruption using physical methods e.g. a cell sieve (Freshney, 1994; Nicholson, 1985).

6.4.1. Tissue Explants

For the explant method, using sterile scalpels and/or scissors, each tissue was cut into minute pieces (<0.5 cubic centimetre) and transferred to either 25 or 75 cm^2 tissue culture flasks or 24-well culture plates containing a minimum volume of cell culture medium. The explanted tissues were incubated at various temperatures in the range 20-30^{o}C. In addition, tissues were also squashed onto the substrate and allowed to adhere to the surface for 3 hours prior to the addition of a minimum of growth medium. This method ensured to ensure that the tissues were in direct contact with the substrate to facilitate cellular outgrowth.

6.4.2. Enzymatic Digestion

For enzymatic digestion, each tissue was digested (at 4^{o}C, room temperature (22-24^{o}C, or 28-30^{o}C) with a specific enzyme solution for various time periods in an attempt to release single cells from the tissue. The following enzymes were trialled:

- 0.25% (w/v) trypsin (Difco Trypsin 1:250 Cat # 0152-15) in hepes buffered saline, adjusted to pH 7.2 with 1N sodium hydroxide, filtered (22 µm) and sterility checked by incubation for 24 hours at 37^{o}C prior to being stored frozen (-20^{o}C).

- 0.45% (w/v) trypsin

- 2.4U/mL dispase

- 200U/mL collagenase Type I, II or IV

- Trypsin/EDTA medium: 0.25% (w/v) trypsin (Difco trypsin 1:250 Cat # 0152-15) in hepes buffered saline, adjusted to pH 7.2 with 1N sodium hydroxide, filtered (22 µm) and sterility checked by incubation for 24

hours at 37^{0}C prior to being stored frozen (-20^{O}C). On thawing for use, sterile 0.1% (w/v) EDTA in water was added.

The tissue digest, containing any released cells, was centrifuged to pellet the cells which were then transferred to 25 or 75 cm^{2} tissue culture flasks containing cell culture medium.

6.4.3. Physical Tissue Disruption

Single cell suspensions could also be obtained by physical disruption of the tissues. Two methods were used. Firstly, cell sieves (BD-Falcon 70 and 100 µm pore size) were utilised. The diced tissues were forced through the cell sieves using the plunger of a sterile syringe and then the resulting cell suspension was aliquoted into culture flasks. Alternatively, syringes and 18 gauge needles were utilised. For this method the diced tissue was loaded into the syringe and forced through the 18 gauge needle to disrupt the tissue. The disrupted tissue was then placed in culture flasks.

6.5. Primary Cultures

6.5.1. Culture Medium

Using established techniques (Nicholson BL, 1985), primary cell cultures were initiated using culture medium which previously has been shown to support growth of cell cultures derived from other marine fish species (e.g. Begar et al. 1997; Chi et al., 1999; Fernandez et al., 1993).

Initial culture media combinations used included:

- 1X and 2X concentration Eagle's minimum essential medium with Earle's salts (EMEM) (Gibco-BRL) supplemented with 10mM hepes buffer, 2mM glutamine, 20% (v/v) foetal bovine serum 50µg/ml gentamycin sulphate and 5µg/ml amphotericin B.

- 1X and 2X Medium 199 (M-199) (ICN Biomedicals Inc.) supplemented with 10mM hepes buffer, 2mM glutamine, 20% (v/v) foetal bovine serum 50µg/ml gentamycin sulphate and 5µg/ml amphotericin.

- 1X and 2X Leibovitz (L-15) (Gibco-BRL) supplemented with 10mM hepes buffer, 2mM glutamine, 20% (v/v) foetal bovine serum 50µg/ml gentamycin sulphate and 5µg/ml amphotericin.

Double strength (2X) media were used in the event that cells from SBT tissues required hypertonic conditions for normal function. Subsequently, since these media did not support long-term cultures, other medium types were used, with various growth promoters. In addition, the media were buffered using different buffering systems, e.g. bicarbonate/CO_2 (5% CO_2 and 95% air) or phosphate buffer in normal atmosphere (without increased levels of CO_2). The different media combinations used in this study are shown in table 1.

It is well established that conditioned medium contains factors which can promote cell growth. Conditioned medium obtained from cell cultures of another marine fish species (pilchard) was also used on some of these primary cultures of tuna cells. In addition, previous methods used to establish other marine fish cell cultures (Japanese studies) have included culture medium of higher osmolarity and thus 5% NaCl was added as a supplement to L-15 and EMEM media to determine whether this would provide any advantage.

6.5.2. Medium Supplements

Other medium supplements, such as pyruvate, non-essential amino acids, human fibroblast and epithelial cell growth factors, heparin and glucose, and agents, e.g. nickel salts, that encourage cellular transformation were also added to some cultures.

Table 1. Summary of culture media types evaluated

#	Basic Medium	Buffer	Serum	Other
1	EMEM	20 mM hepes	10% FBS	
1	EMEM	20 mM hepes	10% tuna	
2	EMEM	bicarbonate	10% FBS	
2	EMEM	bicarbonate	10% tuna	
3	EMEM	Tris-HCl	10% FBS	
3	EMEM	Tris-HCl	10% tuna	
4	Hely-2	bicarbonate	10% FBS	
4	Hely-2	bicarbonate	10% tuna	
5	BME	bicarbonate	10% FBS	
5	BME	bicarbonate	10% tuna	
6	HMEM	Tris-HCl	10% FBS	
7	DMEM	bicarbonate	10% FBS	
8	L-15	bicarbonate	10% FBS	
9	L-15 + 5% NaCl	phosphate	10% FBS	L-15 plus 5% extra sodium chloride
10	EMEM	bicarbonate	10% FBS	Conditioned medium: 50% EMEM, and 50% EMEM growth medium removed from pilchard cell cultures and filtered prior to use
11	EMEM + 5% NaCl	20 mM hepes	10% FBS	EMEM plus 5% extra sodium chloride
12	Thermo Trace Xten™ Go	No additional buffer	1 and 2% FBS	A highly defined, enriched culture medium requiring no serum as a source of growth factors. Low

				concentrations of FBS were used to assist primary culture
13	RPMI	bicarbonate	10% FBS	
14	EMEM	bicarbonate	15% FBS	
15	M-199	bicarbonate	15% FBS	
16	L-15	bicarbonate	15% FBS	
17	EMEM	20 mM hepes	5% FBS and 5% tuna sera	
18	EMEM	bicarbonate	5% FBS and 5% tuna sera	
19	HMEM	bicarbonate	5% FBS and 5% tuna sera	
20	Hely-2	bicarbonate	5% FBS and 5% tuna sera	
21	BME	bicarbonate	5% FBS and 5% tuna sera	

All media types were supplemented with glutamine, antibiotics (50 µg/ml gentamycin sulphate, penicillin, streptomycin, 5 µg/ml amphotericin B), fungizone and non-essential amino acids (NEAA). On occasions some medium was supplemented with non-essential medium 100X concentration)

Legend:
EMEM: Earle's minimum essential medium (Gibco cat #: 41500-018)
BME: Basal medium Eagle (Gibco cat #: 50120-100)
DMEM: Dulbecco's modified Eagle medium (Gibco cat #: 12100-061)
HMEM: Hank's minimum essential medium Tris/HCl buffer (Gibco cat #:41200-080)
L-15: Leibovitz's L-15 medium (Gibco cat #:41300-070)

6.5.3. Incubation Temperature

The body temperature of most fishes remains close to that of the surrounding water, because heat is lost directly into the water during respiration; these fish are poikilothermic. However, in some fishes, such as SBT, a special network of fine veins and arteries called the rete mirabile provides a thermal barrier against loss of metabolic heat. SBT are capable of maintaining their body temperature at several degrees above ambient temperature. Thus a range of temperatures (15, 20, room temperature [22-24^{o}C], 25, 28^{o}C) was used in an attempt to determine the optimum temperature for establishing and maintaining SBT cell cultures. Low incubation temperatures were regulated using cool temperature incubators (e.g. Termaks KPB series 6395 CO_2 incubator) and the higher temperatures were maintained using normal heating incubators/environmental cabinets.

6.5.4. Cell Culture Maintenance

Following seeding of processed tissues into culture vessels, the cultures were incubated at a specified temperature (see section 6.5.3.) overnight and, on the following day, examined by light microscopy for the presence of microbial contamination and cell attachment. Clearly, the occasional contaminated cultures were discarded and all other cultures were maintained under temperature-controlled conditions. Within 24 hours incubation, adherent cells would have attached and, to remove debris from cultures with excessive amounts of red blood cells, tissue debris, unattached cells etc. the culture medium in these selected cultures was replaced with fresh medium and returned to their original incubation temperature. To monitor cell spreading, growth and replication, primary cultures were examined on a regular basis, using an inverted light microscope. As the cells metabolise, nutrients become depleted and metabolites affect the pH of the medium turning it acidic (depending on the buffering capacity of the culture medium used). To ensure optimal culture (nutritional and biochemical) conditions, culture medium was replaced on primary cultures on a regular basis, as determined by changes in the appearance (e.g. colour) of the medium.

6.6. Secondary Cultures

For those primary cultures that demonstrated cellular replication by growing to form a confluent monolayer, a procedure of sub-cultivation was undertaken. Monolayers were sub-cultured using a trypsin-versene solution (Gibco trypsin-versene solution 0.25% in PBSA cat # 15400-054).

Growth medium was decanted from the culture flasks and the cell monolayer gently rinsed using PBSA solution (to reduce the concentration of trypsin inhibitors – protein – and divalent cations), at room temperature, which was then discarded. A minimum volume of trypsin-versene solution was added to the culture flasks and incubated at room temperature for varying times of between 10 and 25 minutes, depending on the origin of the cells. Following cell detachment from the substrate, a single cell suspension of the cell cultures was obtained which was aliquoted into new culture flasks (normally at a split ratio of 1:2) with fresh culture medium.

In the event that the cells may be hypersensitive to the relatively harsh enzyme, trypsin, an alternative, non-enzymatic method was used to release cells from the substrate. Growth medium was decanted from the cultures which were then rinsed with PBSA (Ca2+ and Mg2+ free PBS). Fresh, cold PBSA was added to the cultures which were then incubated at 4OC to release cells from the plastic substrate. The resulting cell suspension was aliquoted into fresh medium.

6.7. Routine Maintenance of Cultures

Cultures were examined on a weekly basis and culture medium was changed as necessary, based on presence of cellular debris or reduced pH. Confluent monolayers were sub-cultured at a split ratio of 1:2. Monolayers were sub-

cultured using a trypsin-versene solution (Gibco trypsin versene solution 0.25% in PBSA). Single cell suspensions were obtained by treatment, at room temperature (22-24^{o}C) with a trypsin-versene solution for approximately 10 minutes.

Similar to the generation of secondary culture, growth medium, containing 10% (v/v) foetal bovine serum, was decanted from the flask and the cell monolayer gently washed using PBSA solution, at room temperature, then discarded. A minimum volume of trypsin-versene solution was added to the flask on the opposite side of the flask to the cell monolayer and allowed to cover the monolayer. The monolayer was rinsed in this solution with gentle rocking of the flask. The cell monolayer was exposed to trypsin-versene until, with gentle tapping of the flasks, the cells detached from the plastic substrate. Trypsinisation times varied depending on the cell type, age of the cell monolayer and the cell density. Exposure to trypsin-versene never exceeded 30 minutes. After the cells detached, excess (10 mL) growth medium was added immediately and the cells were gently aspirated using a 10 mL pipette to obtain a single cell suspension. A split ratio of 1:2 was used to establish new cultures. Cultures which did not thrive were eventually terminated.

Following an incubation period of several weeks, cultures were culled according to their capacity for cell replication until those cultures demonstrating continuous cell replication and the appropriate culture conditions were identified.

6.8. Tuna Serum Analysis

Biochemical analysis of SBT serum was undertaken by **Thermo**Trace. This testing coincided with a new “batch” of FBS purchased by AAHL and used for these experiments.

6.9. Cell Proliferation Assay

There are a number of cell proliferation assays developed in recent years some of which have been developed into commercial kits. Few, if any, have been used on fish cell lines or cultures of other aquatic species (Mulford et al., 2001; Williams et al., 2003). For this project, in order to determine whether SBT cell populations maintained in vitro contained any proliferating cells, a commercial kit (Roche Cat. No. 1 299 964) was adapted for use, as described previously (Williams et al., 2003).

By use of this kit, actively replicating cells are identified by an immunocytochemical assay for the detection of 5-bromo-2’-deoxy-uridine (BrdU) incorporated into cellular DNA using a mouse monoclonal antibody. 5-bromo-2’-deoxy-uridine is an analogue of the DNA precursor thymidine and, when present in the culture medium, is incorporated into DNA of replicating cells during the S (synthesis) phase of the cell cycle. Thus the BrdU-specific monoclonal antibody will bind to BrdU-substituted-DNA which can be localised in the nucleus using immunocytochemical techniques.

The assay was performed as per manufacturer's instructions with some modifications, to make the assay more appropriate for finfish cells.

Finfish cell lines used for the assay, included rainbow trout gonad (RTG-2), epithelioma papulosum cyprini (EPC) and pilchard liver (PL). Finfish cell cultures were grown in 8-well chamber slides (Nalge Nunc International Lab-Tek) and processed at 1 or 2 days post-seeding, when the cultures are actively replicating and have reached approximately 70% confluency.

Three steps of the Roche method were modified to make the assay more suitable for finfish cell cultures.

To accommodate finfish cell lines, lower incubation temperatures (15-26^{o}C) were used during the cultivation period and when the cell cultures were incubated (for 30-60 minutes) with BrdU reagent.

A number of fixatives (ethanol, methanol, formaldehyde, glutaraldehyde) were trialled for use with the aquatic animal cell cultures and the fixative of choice was acetic acid (1 part)/ethanol (3 parts) at room temperature, instead of the recommended ethanol fixative at -20^{o}C. The kit contains reagents, which are compatible with ethanol fixation, but also appears to work well using acetic acid/ethanol fixation.

On completion of the assay, the cell cultures were counterstained with either Mayer's haematoxylin (Dako, Lillie's modification) or Diff-Quik II counterstain (Lab Aids Pty. Ltd, modification of Wrights stain).

BrdU assays using SBT cell cultures, (48 hours post-culture) derived from liver tissue and using RTG-2 cell cultures as positive controls were undertaken.

Briefly, cell cultures were incubated with the BrdU labelling medium for 2 hours, washed three times and fixed at room temperature, as described above. The fixed cultures were then exposed to the working solution containing the anti-BrdU monoclonal (mouse) antibody, as recommended, followed by washing, incubation with anti-mouse-Ig-alkaline phosphatase solution. After further washing, the cultures were exposed to the colour-substrate solution, washed and counterstained prior to mounting for examination by light microscopy.

6.10. Transformation Agents

There are several agents that could be used to encourage transformation (Crane, 1999). In this study, those agents that have been successful in other systems were utilised and included:

- Basic fibroblast growth factor (BFGF) used at 10 and 20 ng/ml final concentration (Sigma Fibroblast growth factor-Basic; Cat # F 0291).

- Nickel Chloride Hexahydrate (Sigma Cat # N-6136) used at 250 µg/ml and 50 µg/ml final concentration

- Nickel Sulfate hexahydrate (Sigma Cat # N-4882) used 250 µg/ml and 50 µg/ml final concentration

7.0. RESULTS/DISCUSSION

7.1. Literature Review

The literature review on marine finfish cell lines/cultures revealed that research on the development of fish cell lines commenced in the 1950s and in the last ten years there have been several publications on the development and establishment of continuous cell lines derived from marine fish species, including gilt-head seabream, *Sparus aurata* L. (Bejar et al., 1997), Asian seabass, *Lates calicarifer* (Chang et al., 2001), grouper, *Epinephelus coioides* (Chi et al., 1999), Japanese knife jaw, *Oplegnathus fasciatus*, a kelp (*Epinephelus moara*)/red spotted grouper (*E. akaara*) hybrid, greater amberjack, *Seriola dumerili* (Fernandez, 1993), turbot, *Scophthalmus maximus* L. (Fernandez-Puetes et al., 1993), Pacific herring, Clupea harengus pallasi (Ganassin et al., 1999), African catfish, *Clarius gariepinus* (Kumar et al., 2001), Japanese flounder, *Paralichthys olivaceus* (Meguro et al., 1991; Tong et al., 1997), sea perch, *Lateolabrax japonicus* and red sea bream, *Pagrosomus major* (Tong et al., 1998). In addition, staff at the AAHL Fish Diseases Laboratory (CSIRO Livestock Industries, Geelong) have recently established two pilchard (*Sardinops sagax neopilchardus*) cell lines, as part of FRDC Project # 99/226 (Williams et al., 2003).

Table 2 provides a summary of the methods used and significant findings from this literature review. In all of the publications a range of fish ages (embryo, larvae, fry, juveniles <1 year old) and sizes was used to generate the cell lines. It is interesting to note that the fish tend to be relatively young (<1-year-olds). In addition, there was the usual range of fish tissues, cell culture media and incubation temperatures used. There was nothing unique or unusual in any of the publications and the research plan for the development of continuous cell lines of SBT took into account all the major variables associated with development of marine fish cell lines, to date. Clearly, as a source of material for establishing cell cultures, fish should be as young as possible (Nicholson, 1985).

Table 2. Summary of Literature Review on Development of Marine Fish Cell Lines

Cell line	Fish species	Age of fish	Culture medium	Tissue (dissociation method)	Incub^{n} Temp.	Reference
FF-11	*Paralichthys olivaceus*	1 yr	L-15 20% FBS	Fin (trypsin/20^{o}C/20 minutes)	20^{o}C	Meguro et al., 1991
SHK-1	*Salmo salar*	1 yr	L-15 5% FBS	Kidney (collagenase)	20^{o}C	Dannevig et al. 1995
YTK	*Seriola quinqueradiata*	<1 yr	EMEM+ NaCl	Kidney (trypsin/RT/10 minutes)	20^{o}C	Watanabe et al. 1981
RSBF	*Chrysophrys major*	fry	EMEM+ NaCl	Whole fry (trypsin/RT/10 minutes)	20^{o}C	Watanabe et al. 1981
SBK	*Lateolabrax*	<1 yr	EMEM+	Whole fry	20^{o}C	Watanabe

	japonicus		NaCl	(trypsin/Rt/10 minutes)		et al. 1981
JSKG	*Oplegnathus fasciatus*	Not known	L-15 20% FBS	Gonads (trypsin/5°C/o-n)	25°C	Fernandez et al. 1993
KRE	*Epinephalus* sp.	embryo	L-15 20% FBS	Embryo (trypsin/5°C/o-n)	30°C	Fernandez et al. 1993
PAS	*Seriola dumerili*	Not known	L-15 20% FBS	Skin (trypsin/5°C/o-n)	25°C	Fernandez et al. 1993
TV-1	*Scophthalmus maximus*	fry	EMEM 10% FBS	Whole fry (explant)	20°C	Fernandez-Puentes et al. 1993
FG-9307	*Paralichthys olivaceus*	<1 yr	L-15 20% FBS	Gill (explant) (NB. heart, kidney, liver, spleen did not grow well)	20°C	Tong et al. 1997
SAF-1	*Sparus aurata*	Not known	Ham's F12/DMEM	Caudal fin Explant	25°C	Bejar et al. 1997
SPH	*Lateolabrax japonicus*	<1 yr	L-15 then EMEM 10% FBS	Heart (explant)	20°C	Tong et al. 1998
SPS	*Lateolabrax japonicus*	<1 yr	L-15 then EMEM 10% FBS	Spleen (explant)	20°C	Tong et al. 1998
RSBF	*Pagrosomus major*	<1 yr	L-15 then EMEM 10% FBS	? (explant)	20°C	Tong et al. 1998
GF-1	*Epinephelus coioides*	<1 yr	L-15 20% FBS	Fin (trypsin/EDTA/ 4°C/30 minutes)	28°C	Chi et al. 1999
PHL	*Clupea harengus pallasi*	larvae	L-15 15% FBS	Whole larvae (explant)	20°C	Ganassin et al. 1999
GK	*Epinephelus awoara*	<1 yr	L-15 15% FBS	Kidney (trypsin/EDTA/ 4°C/60 minutes)	28°C	Lai et al. 2000
GL	*Epinephelus awoara*	<1 yr	L-15 15% FBS	Liver (trypsin/EDTA/ 4°C/60 minutes)	28°C	Lai et al. 2000
SF	*Lates calcarifer*	fry	EMEM +NaCl 15% FBS	Whole fry (trypsin/25°C/15 minutes)	25°C	Chang et al. 2001
LJES1	*Lateolabrax japonicus*	embryo	DMEM+ various supplements	Physical disruption of embryo	25°C	Chen et al. 2003
PL	*Sardinops sagax neopilchardus*	<1 yr	EMEM 10% FBS	Liver (trypsin/RT/10 minutes)	22°C	Williams et al. 2003
PH	*Sardinops sagax neopilchardus*	<1 yr	EMEM 10% FBS	Heart (trypsin/RT/10 minutes)	22°C	Williams et al. 2003
FEC	*Paralichthys olivaceus*	embryo	DMEM+ various supplements e.g. bFGF and fish serum	Physical disruption of embryo	24°C	Chen et al. 2004

7.2. Primary Cell Cultures

7.2.1. Tissue Processing

Most tissues were relatively easy to dice. The muscle tissues (e.g. heart) were, not surprisingly, tougher than other tissues. In contrast, it was noted during processing that the liver tissue had undergone a significant change in that it had become “rubbery” which was viewed as some type of deterioration. This observation indicated that, in future preparations, liver tissue should be the first tissue processed and thus liver was always processed as soon as possible.

It appeared that tissues tended to be resistant to enzymatic treatment, requiring 30-60 minute periods before tissue disruption was evident. Based on these observations different enzymes (in addition to 0.25% trypsin, trypsin/0.1% EDTA, 0.45% trypsin, collagenase Type I, II, IV and dispase) were used and enzymatic treatment was undertaken at various temperatures.

The use of dispase as a dissociating enzyme yielded variable results and its use was discontinued. The use of a higher concentration (0.45%) trypsin for shorter treatment times, in an attempt to yield higher numbers of cells from the treated tissues, was trialled. While tissue dissociation appeared to be more efficient the number of viable cells did not appear to increase or, indeed, appeared to decrease with the exception of kidney tissue.

Relative to the amount of tissue used, the absolute numbers of cells per culture vessel were surprisingly low. The low yield of viable cells indicated that the enzymatic treatment was inefficient, either not effective at releasing cells from the tissues or was too severe resulting in cell death. Additional (non-enzymatic) methods used to process the tissues included the use of explants and the use of cell sieves (70 and 100µm pore size). However, these procedures were not successful in producing primary cell cultures; enzymatic processing of the tissues was more efficient (i.e. yielded higher numbers of cells/culture and a higher number of cultures of growing cells) than the physical methods used to process the tissues.

Of the enzyme treatments used, 0.25% trypsin/0.1% EDTA has been shown to be consistently the most efficient method for tissue processing, yielding significantly more viable cells than any other method used to date.

There were no noticeable affects when different temperatures were used during the enzymatic treatments. Indeed, the use of 0.25% trypsin/0.1% EDTA at 4^{o}C with an overnight incubation yielded encouraging results and has been continued.

7.2.2. Microscopic Observations

For the vast majority of experiments (approximately 24 tissue samplings were undertaken throughout the course of this project), microbial contamination was not evident, demonstrating that the methods used for tissue collection,

dissection and processing had proceeded successfully. In addition, some cell attachment had occurred, especially in the trypsin-treated tissues. However, this observation may be due merely to the small amount of tissue present allowing more efficient examination of these cultures than with the explant cultures which contained greater amounts of tissue which could easily obscure examination.

Of further interest, it was noted in the initial experiment that cells released from the heart tissues were undergoing rhythmic contractions, typical of heart cells in culture, thus demonstrating that the cells were viable and exhibiting normal function. Thus, to this stage of processing, the cells had remained viable.

For each sampling approximately 100-200 cultures, maintained under various conditions, were set up. These cultures were examined, by light microscopy, on a regular basis (2-3 times per week) and maintained (medium change and/or sub-cultured), as needed.

During the first two weeks of culture, it was noted that tuna cells of various morphological types were present in most cultures. Examples of typical cell cultures are shown in figures 2 and 3.

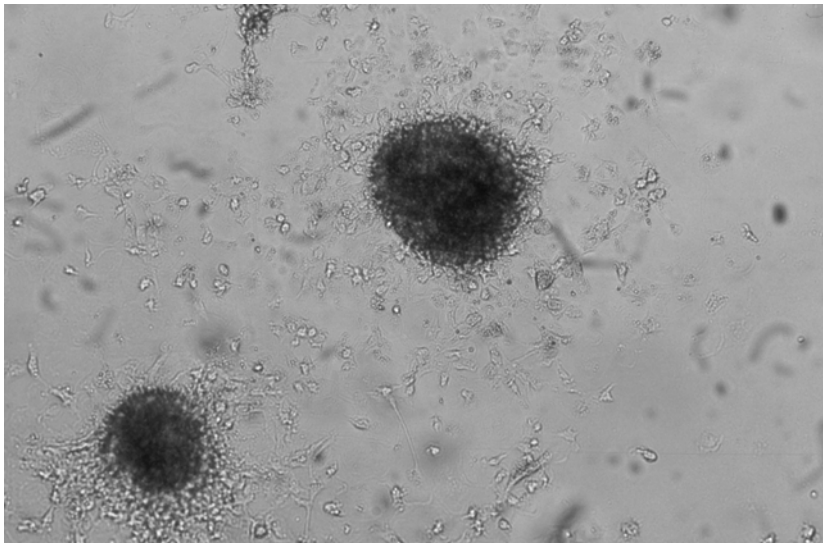

Figure 2. Photomicrograph of 16-day-old primary cell culture derived from SBT spleen tissue treated with 0.25% trypsin and incubated in complete L-15 medium at 28^{o}C. Note the cellular outgrowth from the microscopic pieces of tissue which could be due to either migration of cells from the tissue and/or cellular replication.

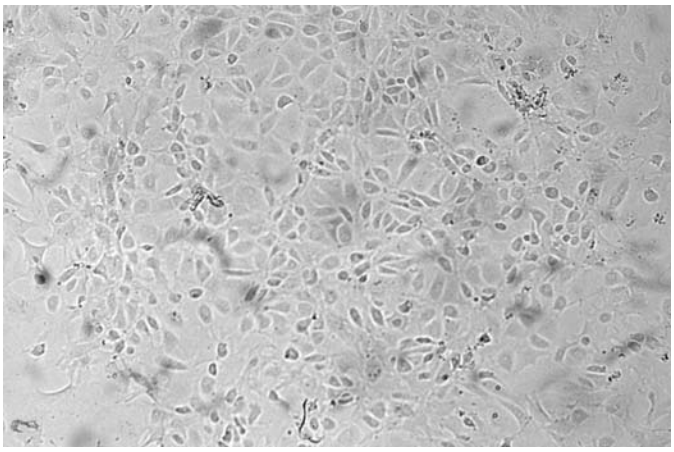

Figure 3. Photomicrograph of a primary SBT cell culture derived from trypsinised liver tissue. Cell culture medium used was M-199 and the culture had been incubated at 22^{o}C for 11 days.

Some cultures grew to full confluency and were sub-cultured successfully (section 7.3).

7.3. Secondary Cultures

7.3.1. Sub-culture Procedure

As the incubation period increased beyond two weeks, many of the cultures tended to deteriorate. In order to retard or reverse this deterioration cultures were provided with fresh culture medium. In addition, for those cultures which had relatively large colonies of cells, these were dispersed using 0.25% trypsin/0.1% EDTA. However, these strategies were not totally successful and, for most of the experiments, most of the cultures deteriorated beyond recovery. A few cultures have persisted for relatively long periods of time (several weeks), grown to full confluency (figure 4) and have been sub-cultured up to 6 times but, to date, cellular transformation to the immortal state has not occurred.

While most cultures have died some important information has been obtained which will assist in future attempts at obtaining long-term healthy cultures from younger fish.

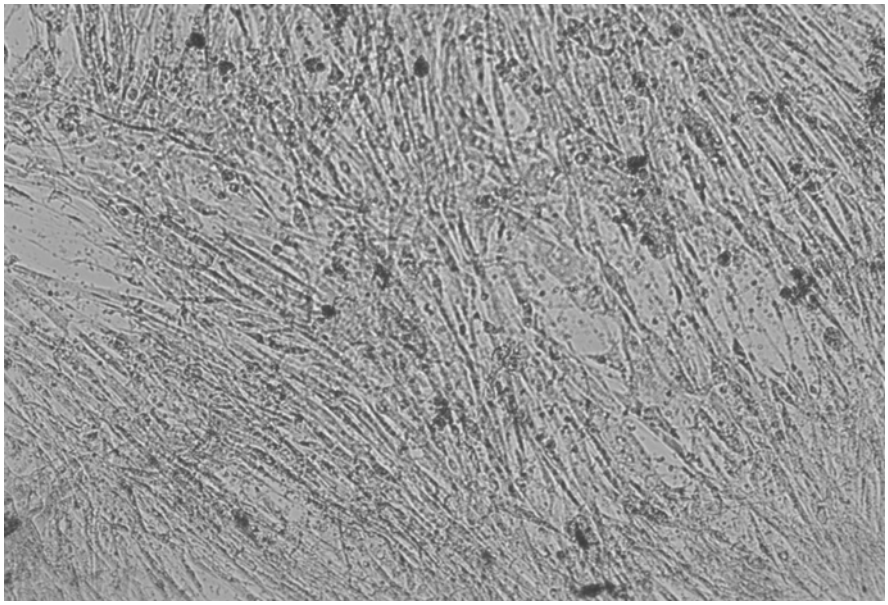

Figure 4. Photomicrograph of SBT spleen cell culture. Primary cultures were established from tissue treated with 0.25% trypsin and grown in Medium 199 at 25^{o}C. Following sub-culture, the secondary culture grew to confluency, as shown. The photomicrograph was taken after 18 days incubation. The vast majority of the cells exhibit a typical fibroblastic morphology.

7.3.2. Incubation Temperature

It was noted that the cultures incubated at 25-28^{o}C appeared to fare better than those at the lower temperatures (either at AFDL or IMVS). Thus, in all later experiments, cultures were incubated at either 25^{o}C or 28^{o}C.

At these higher temperatures, cell attachment to the substrate, cell spreading, growth and replication appeared to progress at higher rates than at the lower temperatures trialled. The general appearance of the cultures (e.g. cellular morphology) was also better.

7.3.3. Culture Medium

Since cultures maintained on the standard culture media (EMEM, L-15) did not survive for prolonged periods (months rather than weeks), other culture media were evaluated to determine whether certain nutritional factors are critical (without having to design custom media) for SBT cell cultures. However, none of the other media used appeared to provide any advantage over the standard media currently used.

In some cases, the use of media supplemented with extra NaCl was detrimental to the cultures and was not continued, even though other cultures with high salt concentrations have persisted. The use of Thermo Trace Xten^{TM} Go medium has showed some promising results with some cultures (derived from spleen) having been maintained for prolonged periods using this medium. However, over the long-term, this medium has not proven to be any more successful than the standard formulations.

Over the period of this project, the use of EMEM as the basic culture medium has yielded the most consistent results with cultures on this medium persisting for longer periods than with other medium types. Currently, the preferred culture medium is:

EMEM supplemented with 10% FBS, 2 mM glutamine, 200 IU/ml penicillin, 200 µg/ml streptomycin and non-essential amino acids. The use of conditioned media did not appear to provide any advantage of the use of fresh medium.

7.3.4. Tuna Serum

In early experiments, it was noted that, in addition to a relatively high number of residual SBT red blood cells, spleen cultures contained a higher number of apparently healthy cells. To determine whether some tuna blood components may be beneficial to establishing SBT cell cultures, SBT serum was obtained (by Mr David Ellis) and was included in the culture medium used for primary culture. Several batches of tuna serum were used and there did not seem to be any reproducible response. It is possible that the tuna serum varied between source animals and there is no basis on which to apply quality control measures. Nevertheless, tuna serum was subjected to biochemical analysis to determine whether there were any obvious differences between tuna serum and foetal bovine serum.

The analysis (table 3) indicates that there are quantitative differences in certain serum components when compared with foetal bovine serum. Whether these differences have any significance relevant to cellular growth and replication is unknown at this time. While the use of certain SBT serum may be beneficial during the early stages of culture experiments yielded variable results and the use of SBT serum was abandoned due to the lack of consistency and quality control of the SBT serum.

7.3.5. Source Tissues

In the twenty-four samplings undertaken, during the course of this project, spleen, liver, kidney and gonad have been the tissues that have consistently produced cell cultures that have been maintained for prolonged periods.

7.3.6. Cellular Proliferation Assay

Primary and secondary cultures appeared to be undergoing cellular replication. Observations, by light microscopy, indicated that the area of the substrate covered by cells was increasing at a steady rate. This increase in substrate coverage could have been due to individual cells spreading to cover more area, cell movement so that cells in multiple layers moved to form a monolayer with increased substrate coverage or the absolute number of cells was increasing i.e. cellular replication was on-going. To determine whether cellular replication was occurring, a cellular proliferation assay was used (Williams et al., 2003).

Table 3. Comparison of SBT and Foetal Bovine Serum Components

Parameters	TUNA SERUM	FBS
pH	7.81	7.22
Osmolality	414 mOsm/ kg	322
Haemoglobin	15.81 mg/dL	15.6
Total protein	63 g/ L	38
Endotoxin	<8.0 EU/mL	0.62
Alkaline Phosphatase	31 U/L	175
Bicarbonate	18 mmol/L	14
Bilirubin	<2 µmol/L	4
Calcium	4.23 mmol/L	3.78
Chloride	177 mmol/L	99
Cholesterol	6.34 mmol/L	<0.9
Creatinine	<0.01 mmmol/L	0.25
Determination of % Oxy Haemoglobin	0.0%	<0.9%
Ferritin	166 ug/L	254
Gamma Glutamyl Transpeptidase (GGT)	<1 U/L	5
Glucose	6.4 mmol/L	7.8
High Density Lipoproteins (HDL)	4.26 mmol/L	0.31
Hormone Profile: Estradiol	<73 pmol/L	567
Hormone Profile: Insulin	<2.0 IU/mL	<2.0
Hormone Profile: Progesterone	2.0 nmol/L	<0.7
Hormone Profile: Testosterone	< 0.3 nmol/L	<0.3
Hormone Profile: Thyroxine (T4)	25.1 pmol/L	26.2
Iron	12 µmol/L	38
Iron saturation	13%	73%
Lactic Dehydrogenase (LDH)	193 U/L	413
Phosphorus (Inorganic)	2.27 mmol/L	3.51
Potassium	2.2 mmol/L	12
Serum Glutamyl Oxalacetate Transaminase	<1 U/L	32
Sodium	214 mmol/L	136
Total Iron Binding Capacity	98 umol/L	53
Transferrin	<0.12 g/L	<0.12
Triglycerides (TG)	4.17 mmol/L	0.79
Urea	0.5 mmol/L	7.4
Uric Acid	0.08 mmol/L	0.16

Using a commercial kit, it was demonstrated that the tuna cell cultures contained replicating cells (figure 5). This direct, objective evidence was important since cellular replication is a prerequisite to the development of continuous cell lines. In order to facilitate the transformation process, various transforming agents were used.

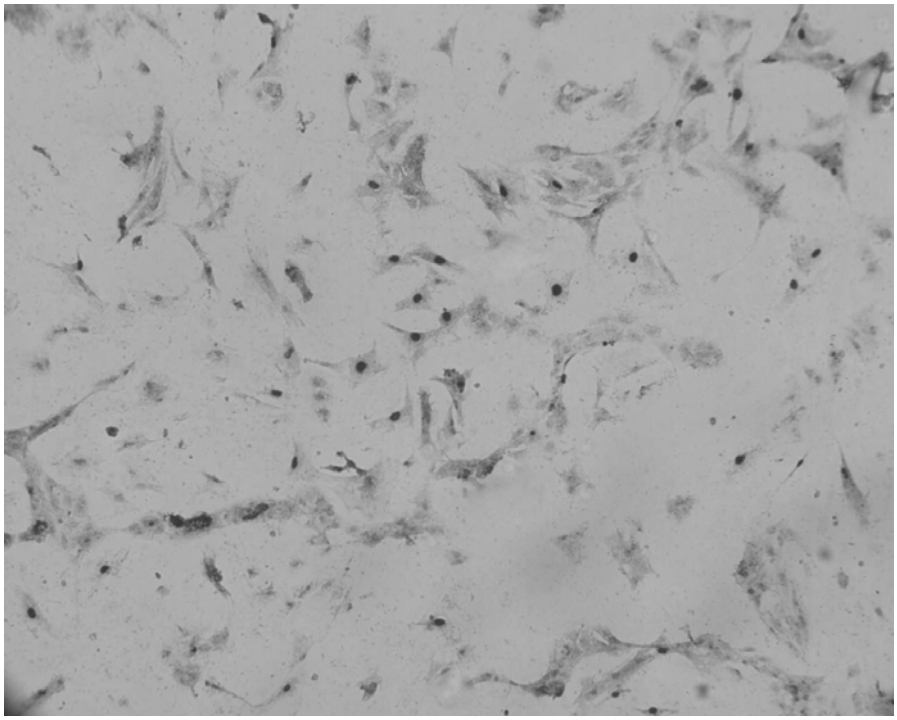

Figure 5. Photomicrograph of SBT liver cell culture labelled with BrdU. Primary liver cell culture was established from tissue treated with 0.25% typsin/0.1% EDTA and incubated at 28^{o}C in EMEM supplemented with NEAA, glutamine, fungizone and gentamycin. Cells were in primary culture for 5 days and then passaged into 8-well chamber slides, incubated for a further 3 days prior to addition of BrdU, fixation and labelling with the monoclonal antibody. Cells that were undergoing DNA synthesis at the time of labelling with BrdU are positively stained (black nuclei in the photomicrograph) by the immunocytochemical technique carried out on the fixed cell cultures. Unstained nuclei would represent cells in either G_1 (presynthetic) or G_2 (post-synthetic) phases of the cell cycle (Crane, 1999).

7.3.7. Nickel ions: Effect On Cellular Transformation

Following primary culture establishment, addition of non-toxic levels of clastogenic agents such as nickel ions (5, 50 & 250 µg/ml nickel sulphate or 5, 50 & 250 µg/ml nickel chloride) to increase the cellular transformation rate were added to cultures demonstrating cellular replication.

Some cultures did not survive for sufficiently long periods to allow evaluation of these treatments. Other cultures were maintained for prolong periods but it was suspected that these underwent senescence, ceased replicating and thus the presence of nickel ions would be ineffective as a transformation agent. No differences between treated and untreated cultures have been observed to

date. However, it is noted that treatment is required for prolonged periods to effect transformation. Previous studies with mammalian cultures indicate that transformation, using these compounds requires continuous exposure of replicating cultures for up to 100 days (Tveito et al., 1989; Trott et al., 1995). Based on results to date, while early cultures contained many cells undergoing DNA synthesis, none of the SBT cell cultures contained significant numbers of replicating cells after incubation periods as long as 3 months.

7.3.8. Fibroblast Growth Factor

Addition of basic fibroblast growth factor (10 & 20 ng/ml bFGF) did not provide any consistent benefit. Certainly bFGF was not detrimental to cultures and its use was continued since other investigators have shown benefits. However, it is likely that previously shown benefits occurred when growth factors such as bFGF were used to treat cultures derived from tissues of very young animals (<1 year old). Again, for any benefit, cultures need to be actively replicating for prolonged periods (> 3 months).

7.3.9. Use of Tumours

Two putative tumours were received and processed through to primary cultures. While these cultures looked promising initially (figure 6), they could not be sub-cultured easily and there are no surviving cultures from these experiments. The use of tumours for generating continuous cell lines is a promising approach and should be continued. In the future, it would be useful to confirm the identity of any perceived abnormal growths/tumours to determine whether culture should be attempted.

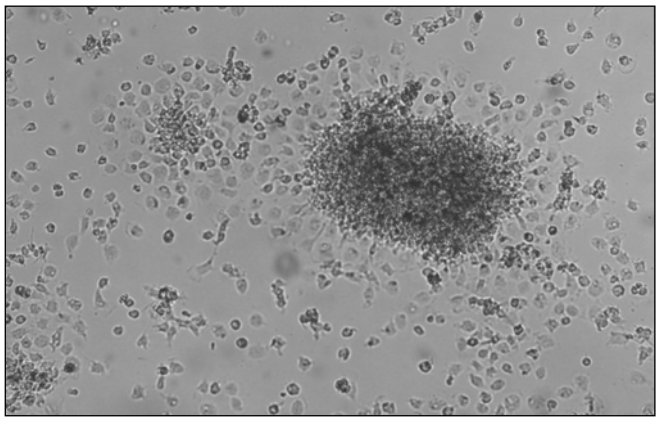

Figure 6. Photomicrograph of tumour explant at 7 days post-culture

7.3.10. Results Summary

During the course of this project, tissues have been sampled from over 40 SBT on twenty-four different occasions. On every occasion, establishment of primary cultures was successful with little, or no, microbial contamination.

Spleen, liver and gonad tissue dissociated with 0.25% trypsin/0.1% EDTA at room temperature for 45 minutes resulted in higher cell yields than other treatments and to-date this treatment is the method of choice for establishing primary cultures. This solution is also used for dissociating cell monolayers for sub-culturing purposes. Overall primary and secondary cultures have comprised of predominantly fibroblastic-like cells.

Results to date indicate that primary cultures of SBT cells are better established at higher (e.g. an incubation temperature of 25-28^{o}C) temperature rather than at lower temperatures (15-22^{o}C).

While media of higher osmolarity appeared, in the main, detrimental, standard culture media such as EMEM supplemented with FBS appeared to provide sufficient nutrition to allow cultures to be maintained over extended time periods during which the cultures could be sub-cultured between one and three times. Occasionally, there was a culture that can be sub-cultured further. The preferred medium, based on results to date, is EMEM with hepes buffer, glutamine, non-essential amino acids, antibiotics (gentamycin and fungizone) and 10% FBS which has yielded the most consistent results.

Following an initial period of cellular replication, the cultures reach a crisis point (Crane, 1999) at which time they can either die or eventually transform and replicate to form discrete colonies which can be expanded to become the progenitors of continuous cell lines. Despite addition of transforming agents, all cultures have died and have been discarded. It was hoped that the addition of growth factors and transforming agents, such as nickel salts, may facilitate transformation which would result in the formation of discrete colonies of cells, detectable with the naked eye and confirmed by light microscopic examination. However, it is likely that due to the relatively old SBT available (2-5-years old) the cell cultures established were unable to maintain sufficiently high replication rate to facilitate transformation.

Cellular replication assays have been performed on some early cultures and results have shown that cells in these cultures are indeed undergoing DNA synthesis. However, assays on some of the older, persistent cultures indicated that the cultures are undergoing, if at all, very low levels of cellular division. It could be concluded that the fish from which these cultures were derived may be of an age that the cells have a limited growth potential and lifespan, and sourcing younger fish should be considered.

Primary cultures could be sub-cultured, using standard procedures to dissociate the monolayers, and many of the resulting secondary cultures were able to grow to 100% confluency. Some secondary cultures of spleen tissue have demonstrated cell replication and growth to 100% confluency through 7 sub-cultures, over a 5-month period. Unfortunately this behaviour is not the norm and the cultures did not transform. Cells with fibroblastic morphology were predominant in these cultures.

7.3.11. Conclusions

Using the resources available, continuous (immortal) cell lines could not be developed from tissues derived from juvenile (2-5 year old) SBT. Following establishment, primary cultures demonstrated an increase in cell number growing to 100% confluency, could be sub-cultured several times with subsequent cellular replication to 100% confluency and, in this way, could be maintained for several weeks. While significant progress towards the development of immortal cell lines has been made, the vast majority of cell cultures could not be maintained beyond 2 months post-initiation. In order to progress to the next stage – cultures demonstrating indefinite growth potential, prior to the transformation to immortal cell lines – further manipulation appears to be required. It is recognised that young tuna fry would be the most appropriate source of cells for development of immortal cell lines. However, SBT fry were not available to the project. Transforming agents were added to cultures in an attempt to obtain continuous cell lines but, due to the age of the source fish, these agents were ineffective.

8.0. BENEFITS AND ADOPTION

The major planned output from this project was to be the establishment of at least one SBT cell line (an immortal cell line derived from tuna tissue) that could be utilised in studies of a number of processes involved in tuna biology. However, the thrust of this project was to obtain a cell line which was susceptible to known viruses of marine fish and which was amenable as a research and diagnostic tool for tuna virology. Dependent on the success of the project in obtaining a suitable cell line, standard procedures for maintaining the cell line and for the isolation of marine viruses using the cell line were to be developed.

Beyond the scope of this project, but with subsequent consultation with the SBT aquaculture sector (industry and state government), standard procedures for detection and isolation of viral agents would be incorporated into an enhanced health surveillance program for the tuna industry. Thus the SBT cell line, and procedures for maintaining and using it, would be transferred to laboratories involved in providing diagnostic services to the SBT aquaculture industry.

While the development of an immortal SBT cell line has not been achieved, project outputs have benefited other Aquafin CRC projects. For example, technology has been transferred to Aquafin CRC Project 2.2 - Tuna Quality at Flinders University, Adelaide (Dr Kathy Schuller and students). AAHL staff have provided advice, resources and training to Flinders University project staff on:

- Maintenance of fish cell lines
- Applications and methodologies
- Establishment of SBT primary cell cultures

9.0. FURTHER DEVELOPMENT

Through the extensive study undertaken as part of this project, it is clear that manipulation of tissues derived from 2-5 year-old SBT will not generate continuous (immortal) cell lines. However, all the basic culture conditions for the maintenance of tuna cells in vitro have been established. It would be expected that, based on previous studies (see section 7.1), the use of young SBT (less than 1 year-old) or relatively small SBT (less than 1 kg in weight) as a source of cells, and using the culture conditions established here, would facilitate the generation of SBT cell lines.

By using younger fish, the primary cultures would contain cells with a higher potential for prolonged growth and replication compared with cells derived from older fish. Thus the likelihood of obtaining spontaneous transformation would be increased significantly and the use of transforming agents may not be necessary.

If, by using young fish and the methodologies outlined here, development of SBT cell lines is still not straightforward, then this would indicate that further detailed research is required. For example, it should be determined whether there are specific nutritional requirements for tuna cells. While, in the authors' opinion, this is unlikely, there may be some essential nutrients that are not provided by the culture media used in this study. The use of tuna serum as a medium supplement may need to be studied more rigorously.

As suggested previously, cell cultures derived from young fish should be comprised of actively replicating cells that could persist for several weeks or months during which spontaneous transformation may occur. Even if transformation did not occur spontaneously, the maintenance of replicating cultures for several months allows manipulations that would facilitate transformation. These manipulations would include treatment with various growth factors and/or clastogenic agents such as nickel ions.

More drastic measures could include the use of viral oncogenes (Hanahan & Weinberg, 2000) or somatic cell genetics viz. cell fusion and selection of hybrids (Littlefield, 1964; Ruddle, 1981) to facilitate cell line development.

10.0. PLANNED OUTCOMES

Following their development, one application of SBT cell lines would be in an improved health surveillance program for the SBT aquaculture sector. Specifically, the cell lines would provide an enhanced capability for the detection and identification of potential viral pathogens of tuna. Currently, this capability does not exist and thus there is a severe limitation on diagnostic laboratories' capability to provide comprehensive health services to the SBT aquaculture sector.

There are examples from other systems (infectious salmon anaemia virus in Norwegian Atlantic salmon; viral diseases of prawns) in which a lack of cell lines susceptible to viral agents has impeded progress in development of other diagnostic tools (Dannevig et al., 1995, Crane & Bernoth, 1996).

The lack of these tools will impose a limitation on the further development of the SBT aquaculture industry. Any plans to close the life-cycle of SBT should include a capability to select healthy (virus-free) broodstock, eggs and fry. It is accepted internationally that, in other aquaculture systems, the most sensitive and reliable procedures for the detection of virus-free stock involve the use of appropriate fish cell lines (OIE, 2003). Thus the availability of tuna cell lines susceptible to marine viruses will also assist in a research program aimed at closing the SBT life-cycle. The use of cell lines will allow potential brood-stock, caught from the wild, to be screened for existing viral infections. In addition, all phases of the aquaculture operations could be monitored for the presence of viral pathogens as part of a health surveillance program.

Thus specific beneficiaries would include the SBT aquaculture industry sector, as well as government and other laboratories responsible for providing health services to the SBT aquaculture industry. Research providers should also benefit from access to tuna cell lines which, as alluded to, can be used to develop improved diagnostic procedures (e.g. immunoassays and PCR methods for detection and identification of viral pathogens).

11.0. CONCLUSION

Establishment of SBT cell lines, while not achieved as part of this project, should remain a goal of the SBT aquaculture industry. The use of such in vitro systems would provide benefits in the long-term.

Applications of SBT cell lines include their use in health surveillance programs. Apart from their use as diagnostic tools for the detection and identification of viral pathogens, cell lines are extremely powerful research tools. As host cells for the growth and purification of viral pathogens, the use of cell lines facilitates the development of improved diagnostic reagents (e.g. specific antisera, PCR primers) for the identification of viruses (Dannevig et al., 1995). The same holds true for intracellular parasites. Apart from facilitating basic research on these parasites (Crane & Dvorak, 1982), in vitro systems can be used as drug screens for the identification of antivirals and antiparasitics (Schmatz et al., 1986; Crane et al., 1986).

In addition, fish cell lines can be used to monitor aquatic pollution (Bols et al., 1985) and for basic studies of the cellular stress response (Babich et al., 1993; George et al., 2000) and can be applied to toxicological studies and screening for antioxidants (Wright et al., 2000).

The use of fish cell lines provides, where appropriate, an attractive alternative to animal studies that is relatively inexpensive, quantitative, reproducible, objective, reductionistic and both animal and operator friendly.

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Appendix 1. Intellectual Property

No intellectual property was generated as a result of this project.

Appendix 2. Project Staff

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