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Development of a black sea bream fibroblast cell line and its potential use as an *in vitro* model for stress protein studies

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Abstract

A fibroblast cell line (BSF) derived from a caudal fin explant of black sea bream (*Mylio macrocephalus*) was developed. The optimum fetal bovine serum (FBS) concentration for fibroblast cell line growth was found to be 15–20% v/v FBS and the optimum temperature range for growth was found to be 26–30 °C. The fibroblast cells displayed a diverse distribution in chromosome number with two modal chromosome numbers of 48 and 54. Upon acute heat shock (+8 °C) the cells displayed a 4.1 fold increase in hsp70 and this elevation was not prolonged as hsp70 returned to near basal levels following a 6 h recovery period. The effect of the hsp70 inducer L-azetidine-2-carboxylic acid was tested and it was found that at a concentration of 10 mM this inducer caused a 2.3 fold increase in hsp70 levels. The sensitivity of the fibroblast cell line to heavy metal exposure was tested by treatment with Cu^{2+} and it was found that hsp70 was significantly elevated in the presence of micromolar concentrations of Cu^{2+}. The data from this study demonstrates that the established black sea bream fibroblast cell line could serve as a useful *in vitro* model for stress protein studies.

Introduction

The application and use of established fish cell lines have provided for much new information in several areas of fish biology. Studies concerning fish developmental biology (Ma et al. 2001), toxicology (Ferrero et al. 1998), physiology (Hightower and Renfro 1988) and molecular genetics (Alvarez et al. 1991; Hong et al. 1998) and transgenic applications (Chen et al. 2003) have incorporated the use of cell lines. Cell lines have been developed from various commercially important fish species and have been applied as experimental models for assessment of heavy metal toxicity (Ryan and Hightower 1994) and for propagation and studies on fish viruses (Chang et al. 2001; Chi et al. 1999; Kang et al. 2003; Lai et al. 2000, 2001, 2003; Lannan et al. 1984). In recent years, the sea bream has emerged as an important economic fish species in several Pacific and Mediterranean rim countries and presently only cell lines from gilthead sea bream (Bejar et al. 1997) and red sea bream (Tong et al. 1998) have been developed. We have been studying several aspects of black sea bream biology and it is clear that abiotic stress can exert significant changes on physiological function including immune responses (Narnaware et al. 2000), growth regulation (Deane et al. 2002) and osmoregulation (Kelly et al. 1999a, b). Also, it has been found that black sea bream exposed to salinity stress (hypoosmotic – hypersaline) or heat shock display the rapid upregulation of stress proteins belonging to the hsp70 family (Deane et al. 2002). These proteins are important during stress as they play a key role in cytoprotection helping to repair damaged proteins (Geething and Sambrook 1992). Thus far, all of our previous studies on hsp70 regulation in black sea bream have been based on whole animal studies and the use of an *in vitro* model from this fish species would add to our knowledge on the mechanism and regulation of hsp70 expression at the cellular level. The use of a cell line for studies on hsp70 expression would be useful for stress protein expression studies since sufficient cells could be grown relatively easily

and all of these cells would be in a similar condition prior to experimentally induced stress. In this study we describe the development and characterization of a fibroblast cell line from black sea bream and we describe some features of the hsp70 response in these cells.

Materials and methods

Establishment and fibroblast cell line

Healthy juvenile black sea bream (*Mylio macrocephalus*) were obtained from a local fish farm and maintained in laboratory aquaria equipped with seawater recirculation. Fish were kept at a temperature of 18–22 °C and fed *ad libitum*, twice daily with a formulated diet (Woo and Kelly 1995). Fish were acclimated to these conditions for two weeks prior to experiments. At 1 d, prior to tissue removal, fish were transferred to a separate tank containing autoclaved seawater which was supplemented with 1000 U ml^{-1} penicillin (Sigma, USA) and 1 mg ml^{-1} streptomycin (Sigma). The external surface of fish was wiped with a 75% v/v ethanol solution and a small piece of the caudal fin was excised and washed twice in sterile phosphate buffered saline (PBS, Na_2HPO_4 7.82 mM; KH_2PO_4 1.41 mM; NaCl 136 mM; KCl 2.68 mM, pH 7.2) supplemented with 1000 U ml^{-1} penicillin and 1 mg ml^{-1} streptomycin. Caudal fin tissue was cut into small pieces of approximately 1 mm^2 and seeded onto 25 cm^2 culture flasks (Coming, USA). To each flask, 1 ml of Leibovitz 15 (L15, Sigma) complete media supplemented with 20% v/v fetal bovine serum (FBS, Hyclone Laboratories Inc., USA), 200 U ml^{-1} penicillin, 200 μg ml^{-1} streptomycin and 500 ng ml^{-1} amphotericin B (Sigma), were added and the cultures were incubated at 26 °C in an incubator set at 5% v/v CO_2 (Shell Laboratories, IR248). After 24 h incubation, some tissue fragments adhered to the base of the culture dish and an additional 2 ml of L15 complete medium, supplemented with FBS and antibiotics, was added. The culture medium was carefully aspirated at 3–5 d intervals and fresh medium was added. The cultures were periodically observed for morphology and general migration of cells from tissue fragments using an inverted microscope (Zeiss, Axiovert 135) and photographed using a camera (Ricoh XR 2000). When cell monolayers became confluent they were subcultured by removing L15 medium, rinsed briefly in PBS and then disaggregated by adding PBS containing 0.1% w/v trypsin (Sigma)/0.01% w/v EDTA (Sigma), for 1 min with gentle agitation. The trypsin solution was removed and 3.5 ml of fresh L15 complete medium was added to the flask and the disaggregated cells were released into suspension by gentle pipetting. The entire cell suspension was then transferred to a sterile culture flask and incubated as described previously. The cells were subcultured weekly and up to passage 3, epithelial and fibroblast cells were found to coexist in cell preparations but after passage 3 only fibroblasts remained. The subsequent fibroblast cell line was designated as BSF.

Effects of FBS and temperature on fibroblast growth

The effect of varying concentrations of FBS and different incubation temperatures on BSF cell growth was studied. For this purpose fibroblast cell line at passage 60 was tested for growth at three FBS concentrations (10, 15 and 20% v/v) and a range of temperatures (20, 26, 30 and 32 °C). In all experiments the initial cell density was 5×10^4 cells per flask and cell density was measured daily according to previously described methods (Tong et al. 1997).

Chromosome number analysis

The chromosome numbers of BSF cells, at passage 60, were measured using a method previously described (Alvarez et al. 1991), with minor modifications. In brief, BSF cells were incubated in L15 medium supplemented with 1 μg ml^{-1} colchicine (Sigma) for 1 d at 26 °C. After incubation, culture flasks were gently agitated to release cells into medium and the entire culture medium was then carefully decanted and centrifuged at 1000 rpm for 5 min to pellet cells. The supernatant was discarded and the cell pellet was rinsed twice in PBS. After rinsing, the cell pellet was resuspended in 5 ml of a 75 mM solution of KCl and incubated at 26 °C for 30 min. After incubation the cells were pelleted by centrifugation at 1000 rpm for 5 min and 5 ml of cold Carnoy's fixative was added to resuspend cells. A drop of the cell suspension was placed onto a cold glass slide and allowed to air dry. Cells were stained with Giemsa stain (Sigma) for 25 min and then rinsed with distilled water. Cells were observed under oil immersion using a microscope (Nicrophot-FX) and chromosome numbers were counted for each cell.

Heat shock response of BSF cell line

To prepare cells for heat shock experiments two 25 cm^2 culture dishes containing BSF cells at confluence were used. The culture medium in each flask was aspirated and the cells were incubated for 5 min with 1 ml trypsin solution. The trypsin solution was removed and 12 ml of L15 complete media was added to each flask and the cells were dispersed by gentle agitation. The cells from each flask were pooled and 1 ml of preparation was added to individual wells of a 24 well sterile cell culture cluster plate (Costar, USA). Cells were incubated at 26 °C for 4–5 days until the cells became confluent. For heat shock experiments, cells were exposed to control temperature (26 °C) or an acute heat shock at 34 °C for 2 h, or an acute heat shock (34 °C for 2 h) plus recovery at 26 °C for periods of 2, 4, 6 and 12 h. After stress exposure regimes were complete the culture medium was removed and cells were lysed in 0.2 ml of protein extraction buffer (4 M urea, 0.5% w/v SDS, 2 mM PMSF, 10 mM EDTA). Extracts were incubated at 95 °C for 10 min, sonicated for 10 min and then centrifuged at 13,000 rpm for 10 min. The cell extract was measured for total protein content using the dye binding method of Bradford (1976). Samples were read at 595 nm using a spectrophotometer (Milton Roy Spectronic, USA) and protein concentration calculated from a protein standard curve of bovine albumin (Sigma).

Effects of L-azetidine-2-carboxylic acid and $CuSO_4$ exposure on fibroblast hsp70 expression

Fibroblast cells were exposed to the hsp70 inducer, L-azetidine-2-carboxylic acid (Sigma) and $CuSO_4$ (Sigma), in a multiwell plate as described above. To test for chemical induction of hsp70, groups of cells were exposed to L-azetidine-2-carboxylic acid at final concentrations of 0 (control) 0.1, 1 and 10 mM for 4 h at 26 °C and then extracted for total protein. To test for sensitivity to Cu^{2+} cells were exposed to different concentrations of Cu^{2+} (1 nM to 1 mM as $CuSO_4$) for 4 h and then extracted for total protein.

Protein gel electrophoresis and immunoblotting

One dimensional SDS-PAGE was applied to resolve proteins of different molecular size according to the method of Laemmli (1970) using a 4% (stacking) and 12% (separating) polyacrylamide gel. The standard protein used for SDS-PAGE was bovine brain hsp70 (Sigma). For electrophoresis, 1.5 μg of full range rainbow molecular weight marker (Amersham, UK), 0.2 μg of hsp standard protein and a representative protein sample (10 μg) from each treatment group were electrophoresed for 90 min at 100 V followed by transfer to nitrocellulose membrane (Gibco-BRL, USA) for 1 h at 150 V using a Bio-Rad mini kit (Bio-Rad laboratories, USA). After protein transfer the membrane was air dried and then blocked by immersing in PBS, containing 3% w/v skimmed milk powder, overnight at 4 °C. The membranes were rinsed for 1 h in PBS containing 0.05% v/v Tween 20 (PBS-T) and then incubated for 1 h with anti-mouse hsp70 monoclonal antibody (Sigma, cat no: H5147) diluted in PBS-T. The dilution of hsp70 antiserum was 1:2000. The membranes were rinsed for a further 1 h in PBS-T before incubating with antimouse IgG horseradish peroxidase conjugate (Sigma) diluted 1:4000. After a final membrane rinse the bound hsp70 were detected using an ECL development system (Amersham). Immuno-dot blots were used to assess all samples for hsp70. The method used for preparation of immuno-dot blots was essentially as previously described by Deane et al. (2002). Nitrocellulose membranes were prepared for blotting by immersing in 0.1M PBS for 10 min and then fixed into a Bio-Dot microfiltration manifold (Bio-Rad). An aliquot containing 10 μg total protein was added to 100 μl of 0.1 M PBS and samples were added to the wells of the manifold. A vacuum was used to draw the samples onto the membrane and to ensure that all protein sample was drawn onto the membrane, each well of the manifold was washed with a further 200 μl of 0.1M PBS. The samples were fixed onto the membranes by air drying overnight and membranes were probed as previously described and analyzed using a Lumi-Imager workstation (Roche, USA). For each sample the optical density (OD) $\times$ area (mm^2) was quantified using Lumi-Analyst 3.1 software (Roche).

Data and statistical analysis

The hsp70 amounts for each sample were subjected to a one way ANOVA. In order to delineate significance among groups a Student – Newman – Kuels test (Jandel Scientific) was used. Significant differences were accepted if $P < 0.05$ and all data were expressed as mean $\pm$ SEM.

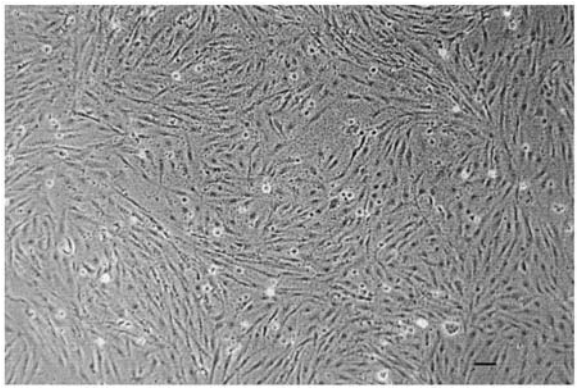

Figure 1. Photomicrograph of black sea bream fibroblast cell line (BSF). Scale bar = 50 μm.

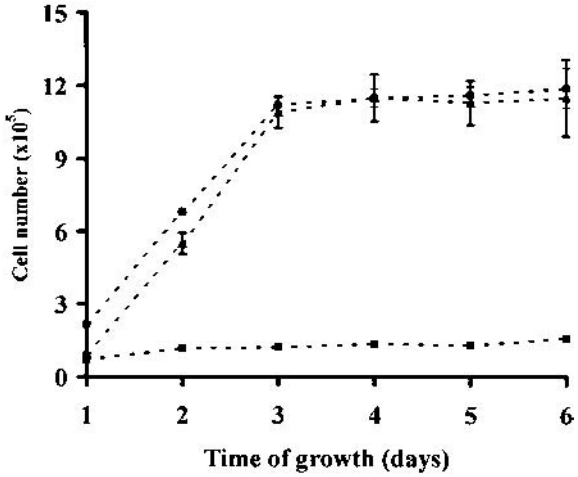

Figure 2. Effects of FBS concentration on the growth response of BSF cell line. Cells were cultured in 10% v/v FBS (- - -■- - -), 15% v/v FBS (- - -▲- - -) or 20% v/v FBS (- - -●- - -). Cell number is represented as number of cells per 25 cm^2 culture flask. Each data point represents a mean value ± SEM (n = 3).

Results

Characterization of BSF cell line

Up to passage 3 a mixture of two cell types were observed to coexist in preparations. The first and dominant cell type were long fibriform cells which were identified as fibroblasts and the other less abundant cell type were short, quadrate cells identified as epithelial cells. After the third passage only fibroblast cells were observed (Figure 1). From an initial 7 primary cultures which were set up, only one could be maintained past 10 passages and this cell line was designated as BSF. The cell line encountered crisis at passage 50 and proliferation was halted. During this time the culture medium was changed frequently to await spontaneous immortalization. The growth rate of BSF cells in varying concentrations of FBS was tested. It was found that cells grew poorly at 10% FBS whereas at 15% and 20% FBS the growth rates were similar (Figure 2). The effect of temperature on growth of BSF cells was studied and it was found that cells grew well at temperatures of 26 °C and 30 °C (Figure 3). Cells grew slowly at 20 °C and 32 °C and it would appear that both of these temperatures were not suitable for BSF cell culture because the growth rate started to decrease at 4 days after cells were seeded onto culture plates. The chromosome number of BSF cells was counted and it was found that these cells exhibited aneuploidy in metaphase and the chromosome numbers were diverse. For BSF cells the chromosome number ranged from 36 to 56 with two observed modal chromosome numbers of 48 and 54 (Figure 4).

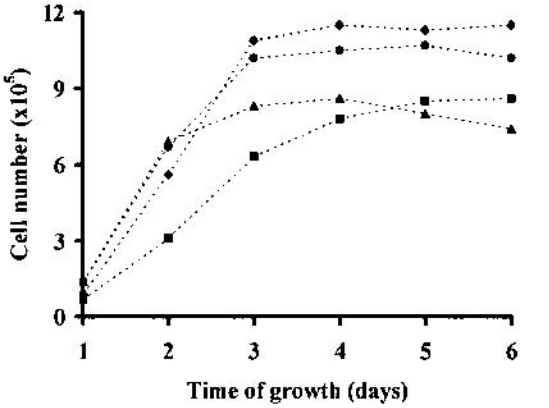

Figure 3. Effects of temperature on growth of BSF cell line. Cells were grown at temperatures of 20 °C (- - -■- - -), 26 °C (- - -♦- - -), 30 °C (- - -●- - -) or 32 °C (- - -▲- - -). Cell number is represented as number of cells per 25 cm^2 culture flask.

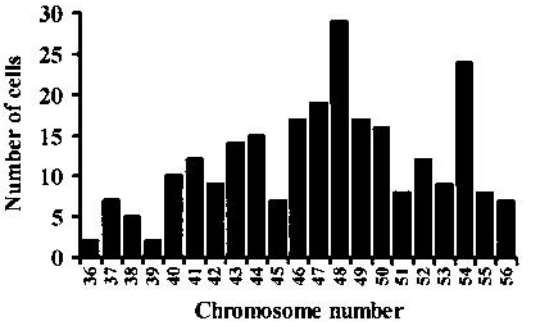

Figure 4. Chromosome number distribution in BSF cell line. A total of 249 cells were stained and counted.

Expression of fibroblast hsp70 during stress response

Using immunoblotting of fibroblast total protein, with an hsp70 monoclonal antibody, we found a single band of 70 kDa migrating to the same size as that of the standard protein (Figure 5A). The levels of fibroblast hsp70 were increased 4.1 fold following an acute heat shock. The elevated hsp70 was not prolonged as a return to near basal levels occurred after a recovery period of 6 h (Figure 5B). The effects of the hsp70 inducer L-azetidine-2-carboxylic acid was tested and it was found that at a concentration of 10 mM a significant increase of 2.3 fold above control levels occurred (Figure 6). The fibroblast cells were also very sensitive to heavy metal exposure since concentration as low as 1 μM Cu^{2+} could cause a significant increase in hsp70 levels (Figure 7). The levels of hsp70 significantly increased 1.7, 1.9, 2.2 and 2.7 fold upon exposure to Cu^{2+} at concentrations of 1, 10, 100 and 1000 μM respectively.

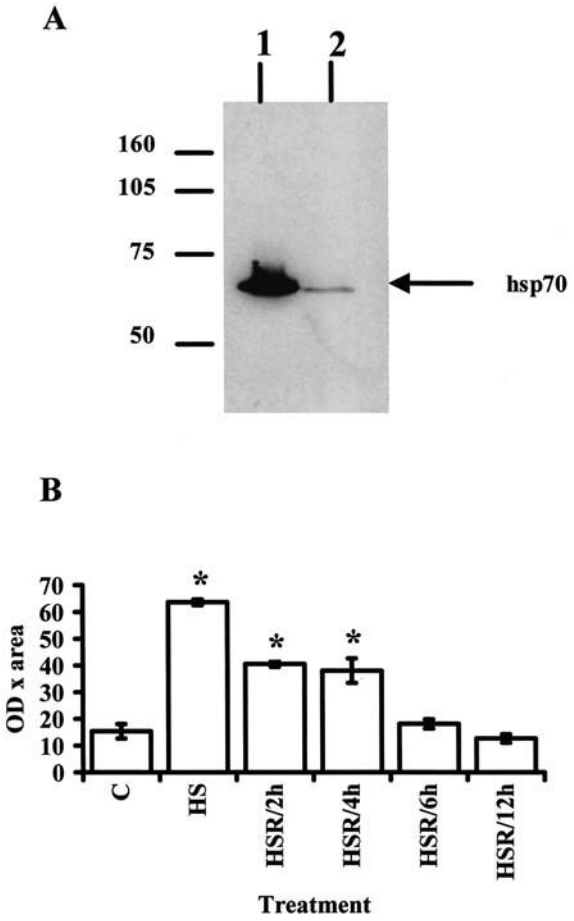

Figure 5. (A) An immunoblot showing presence of hsp70 in cells, 200 ng of hsp70 standard protein (lane 1) and 10 μg of fibroblast total protein (lane 2) were used and the position of molecular markers are indicated in kDa; (B) hsp70 response of BSF cell line following an acute heat shock of +8 °C for 2 h and during a recovery period. The amount of hsp70 was calculated from immunoblots and expressed as OD × area. The values of each test represent a mean ± SEM ($n = 3$) and an asterisk above a bar indicates values which are significantly different from control ($P < 0.05$). The labels on the X axis are as follows C (control cells at 26 °C); HS (heat shocked cells at 32 °C, for 2 h); HSR/2 h (heat shocked cells followed by a 2 h recovery at 26 °C); HSR/4 h (heat shocked cells followed by a 4 h recovery at 26 °C); HSR/6 h (heat shocked cells followed by a 6 h recovery at 26 °C) and HSR/12 h (heat shocked cells followed by a 12 h recovery at 26 °C).

Discussion

In this study we describe the development and characterization of a black seabream fibroblast cell line designated as BSF. The cells were derived from a small piece of the caudal fin and this tissue has been widely used in the preparation of both primary cultures or cell lines of fibroblasts from freshwater and marine fish species including medaka (Komura et al. 1988), northern pike, goldfish, gilthead sea bream (Alvarez et al. 1991; Bejar et al. 1997), red sea bream (Tong et al. 1998) and grouper (Chi et al. 1999). In early subcultures (up to passage 3), both fibroblast and epithelial cells were found to coexist. The fibroblast cell population was observed to grow more rapidly than epithelial cells and after the third generation only fibroblasts remained in culture. Two cell lines from medaka (OL-17 and OL-32) also displayed a similar feature in early cell generations (Komura et al. 1988) and this phenomenon could be explained by the presence of platelet-derived factors, in FBS supplement, which exert potent mitogenic effects on fibroblasts whilst inhibiting epithelial cell proliferation (Freshney 1994). The methods we have described in this study are suitable for the preparations of a fibroblast cell line derived from caudal fin of black sea bream.

We assessed some basic characteristics of BSF cell line encompassing optimal FBS concentration, optimal temperature for growth and chromosome number. The optimal FBS requirement for BSF cells was found to be within 15–20% v/v. This amount of FBS requirement is higher than FBS supplement amount reported for several other fish cell lines. Nine salmonid cell lines (Lannan et al. 1984), two sea perch cell lines (SPH and SPS, Tong et al. 1998), a red sea bream fibroblast cell line (RSBF, Tong et al. 1998), and a predominantly epithelial cell line from Asian seabass (SF, Chang et al. 2001) were maintained in culture me-

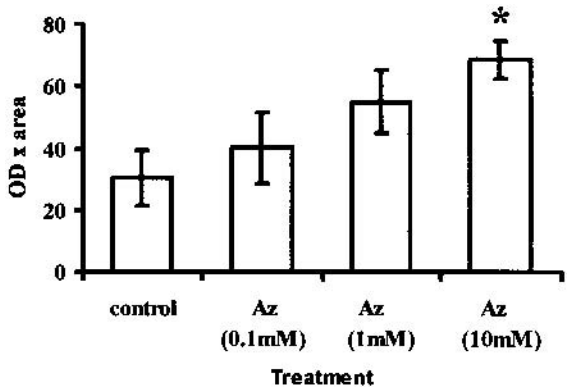

Figure 6. Effect of L-azetidine carboxylic acid (Az) on hsp70 expression in BSF cell line. Three different concentrations of L-azetidine carboxylic acid were tested. The amount of hsp70 was calculated from immunoblots and expressed as OD x area. The values of each test represent a mean ± SEM ($n = 3$) and an asterisk above a bar indicates values which are significantly different from untreated control ($P < 0.05$).

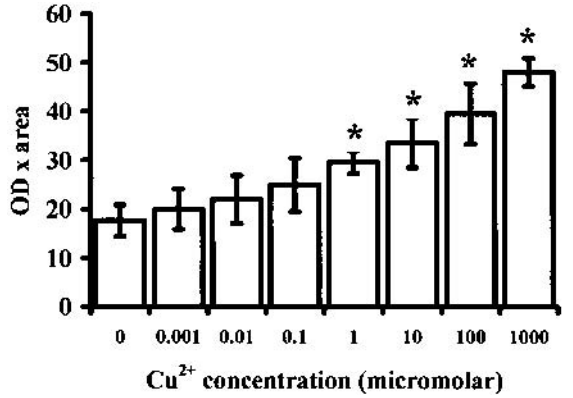

Figure 7. Sensitivity test of BSF cell line to increasing concentrations of Cu^{2+}. The amount of hsp70 was calculated from immunoblots and expressed as OD x area. The values of each test represent a mean ± SEM ($n = 3$) and an asterisk above a bar indicates values which are significantly different from untreated control ($P < 0.05$).

dia containing 10% v/v FBS. It has also been reported that a gilthead sea bream fibroblast cell line (SAF-1) could be cultured at an optimal FBS concentration as low as 5% (Bejar et al. 1997). The higher optimal requirement for FBS supplement may reflect genetic and metabolic differences between BSF cells and cells from other fish species. Although fish serum was not tested, as a supplement, future experiments aimed at using this are certainly warranted. We also tested the optimal temperature for growth of BSF cells and found that optimal growth occurred at temperatures between 26–30 °C. From the available literature it does appear that optimal temperatures for cell line proliferation may be related to whether the cells were originally derived from a 'warm water' or 'cold water' dwelling fish species. Cell lines established from 'warm water' fish require higher temperatures for optimum growth as epithelial cells from grouper (Nicholson et al. 1987) and red sea bream fibroblasts (Tong et al. 1998) displayed optimum growth at 30 °C and fibroblasts from sea perch and grouper displayed optimal growth at 25 °C (Nicholson et al. 1987). Cells lines established from 'cold water' fish appear to have lower optimum growth temperatures as nine cell lines from salmonids showed best growth between 21–24 °C (Lannan et al. 1984). The chromosome number in BSF cells ranged from 36–56 with two observed modal values of 48 and 54. The modal chromosome number of 48 for BSF cells is the same as that reported from other sea bream cell lines (Bejar et al. 1997; Tong et al. 1998) and is identical to the diploid number of sea bream (Bejar et al. 1997). The detection of a second modal chromosome number of 54 was unusual but may not be a unique occurrence as the salmonid cell line CHH-1 displayed a bimodal chromosome distribution (Lannan et al. 1984). Presently it is unclear as to why two modal chromosome numbers can occur in fish cell lines however the diverse chromosome number and the occurrence of aneuploidy are key indicators of immortalized cells (Freshney 1994). The cause of aneuploidy, in the BSF cell line, remains to be elucidated, but may occur as a consequence of chromosome disjoining during cell division.

Several studies have described the use of established cell lines for fish heat shock protein studies. The results from these studies have shown that fish heat shock proteins can be induced by thermal stress (Airaksinen et al. 1998; Chen et al. 1988), anoxia (Ossum et al. 2004) as well as heavy metal exposure (Heikkila et al. 1982; Misra et al. 1989). Of all the heat shock protein families, the hsp70 family bas been most widely studied as a biomarker of stress (Ryan and Hightower 1996) and in our previous studies on sea bream we have assessed the expression profile of hsp70 using *in vivo* approaches (Deane et al. 2002). To extrapolate on these previous *in vivo* studies and to study the expression of hsp70 using an *in vitro* system we first tested whether BSF cells would demonstrate a typical hsp70 response profile, by subjecting cells to an acute heat shock. It was clear that after only two hours of a +8 °C heat shock, a rapid and significant increase of 4.1 fold above basal levels in hsp70 had occurred. The elevated hsp70 levels observed with heat shocked BSF cells was not prolonged as a return to near basal levels was found to occur after a

6 h recovery period, at control temperatures, therefore confirming a role of hsp70 as a stress associated protein in black sea bream fibroblasts. We further tested the hsp70 response of BSF cell line using the known hsp70 inducer, L-azetidine 2-carboxylic acid. Using mammalian cell models L-azetidine 2-carboxylic acid treatment was demonstrated to induce hsp70 transcription in murine embryo fibroblasts (Lee and Seo 2002) and this inducer also increased the synthesis of hsp70 in human erythroleukemia (K562) cells (Fishelson et al. 2001). Similarly, we found that L-azetidine 2-carboxylic acid at a concentration of 10 mM could increase hsp70 levels by 2.3 fold in BSF cells, suggesting a similar mechanism for hsp70 induction exists between BSF cells and mammalian cells. We also tested the sensitivity of black sea bream fibroblasts to heavy metal ion exposure and found that Cu^{2+} at a concentration as low as 1 μM could significantly elevate hsp70 levels. Using a chinook salmon embryo cell line (CHSE-214) Cho et al. (1997) also demonstrated that Cu^{2+} (400 μM) could cause the induction of a 90 kDa stress inducible protein. It is not known if the induction of hsp is mediated via Cu (II) or Cu (I) as the role of cellular oxido-reductases in hsp induction mechanisms is unknown. The responsiveness and sensitivity of the established black sea bream cell line developed in this study suggests that we now have a useful *in vitro* model for further studies on mechanisms of hsp70 regulation in black sea bream.

In summary, we have developed and characterized a black sea bream fibroblast cell line. The cell line is relatively easy to maintain as we have deduced the optimum FBS requirement and growth temperature. Over the past few years we have been studying the effects of hormones on hsp70 expression *in vivo* (Deane et al. 1999, 2000) but due to endocrine axis overlap and crosstalk, firm conclusions about hormone specific effects on hsp70 expression await the application of suitable *in vitro* models. The development of the cell line in the present study may prove to be useful towards these studies.

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