Development of ES cells from Sparus aurata embryos: Preliminary results

Béjar J., Alvarez M.C., Hong Y.

in

Bartley D.M. (ed.), Basurco B. (ed.).
Genetics and breeding of Mediterranean aquaculture species

Zaragoza : CIHEAM
Cahiers Options Méditerranéennes; n. 34

1998
pages 131-139

Development of ES cells from *Sparus aurata* embryos: Preliminary results

J. Béjar*, Y. Hong and M.C. Alvarez***
*Dpto. de Genética, Fac. de Ciencias
Universidad de Málaga, 29071 Málaga, Spain
**Pysiological Chemistry, Biocenter of the University of Wurzburg,
Am Hubland, 97074 Wurzburg, Germany

SUMMARY - Pluripotent embryonic stem (ES) cells represent a cellular link between *in vitro* and *in vivo* genetic manipulation in animals and also have an enormous potential in development studies. To derive ES cells from the gilthead sea bream (*Sparus aurata*) species, seems especially attractive due to its high economic value. To set up the methodology for feeder free cultivation, primary cultures were initiated from blastomeres, isolated from mid-blastula embryos. The media used were empirically designed according to Hong and Schartl (1996). Specific supplements were individually evaluated in terms of improving mitogenic activity and preventing cell differentiation. At present, putative ES cells, as suggested by their special morphology and by the positive staining to alkaline phosphatase, have been cultured for 6 passages. Chromosome analysis reveals a normal karyotype and high survival rate has been obtained subsequent to freezing. The next steps will consist of isolation of single colonies and additional testing of totipotency before being injected into recipient embryos to produce seabream chimeras.

Key words: Embryonic stem cells, transgenic fish, sea bream.

RESUME *– "Développement de cellules souches embryonnaires à partir d'embryons de* Sparus aurata: *Résultats préliminaires". Les cellules souches embryonnaires (CSE) pluripotentes font le lien, à l'échelle cellulaire, entre les manipulations génétiques "in vitro" et "in vivo" chez l'animal, et ont donc un énorme potentiel dans les études sur le développement. Produire des CSE chez la daurade (Sparus aurata) paraît tout spécialement intéressant, à cause de l'intérêt économique considérable de cette espèce. Pour mettre au point une méthode de culture sans cellules nourricières, des blastomères, isolés à partir d'embryons au stade mi-blastula, ont été mis en culture primaire. Le milieu utilisé a été préparé empiriquement, suivant le protocole de Hong et Schartl (1996). Des facteurs additionnels ont été évalués individuellement, en fonction de leur capacité à augmenter l'activité mitotique et à inhiber la différenciation cellulaire. A présent, des CSE putatives (ce que suggère leur morphologie particulière et leur réaction positive à la phosphatase alcaline) ont été cultivées pendant 6 passages. L'analyse cytogénétique a montré que leur caryotype était normal, et des taux élevés de survie ont été obtenus après congélation-décongélation. Les prochaines étapes de ce travail sont l'obtention de colonies uniques et le passage de tests supplémentaires de totipotence, avant l'injection de ces cellules dans des embryons receveurs pour produire des daurades chimères.*

Mots-clés : *Cellules souches embryonnaires, poisson transgénique, daurade.*

Introduction

Embryonic stem (ES) cells are non-differentiated cell lines developed from early animal embryos, which retain their development totipotency after long-term culture

and that under specific conditions can differentiate into diverse cell types (Evans and Kaufman, 1981). The availability to manipulate these cells in culture allows to generate site-directed mutations in their genome. They can be reintroduced into an early host embryo and develop into any kind of tissue. Gametes from chimaeras derived from ES cells will transmit its information to the progeny in a Mendelian fashion, thus representing a cellular link between *in vitro* and *in vivo* genetic manipulation in animals. In addition to this practical application leading to a rapid genetic improvement of livestock, they may have an enormous potential to study cell totipotency and differentiation and can also be envisaged as an attractive strategy for biodiversity preservation.

So far, ES cell lines have been developed in a few mammalian species and are commercially available in mice. However, little is known about the conditions in which they are produced and propagated. The derivation of ES cells from fish species has been partially successful in zebrafish (Collodi *et al.*, 1992) and medaka (Wakamatsu *et al.,* 1994) but with the inconvenient of using feeder layers. In the fish area, an important contribution has been the development of feeder free stable ES cultures in medaka by Hong and Schartl (1996).

Due to the high economic value of the marine gilthead sea bream species *Sparus aurata,* obtaining ES cells seems to be very attractive, since they could be used as an autologous specific system to study gene function by site-directed mutations, as well as for the genetic improvement of this species. The objective of this report is to describe the setting up of the appropriate methodology for feeder free cultivation of seabream embryonic cells in this species.

Material and methods

Primary cultures and subculturing

For each culture, a group of about 50 embryos were disinfected with 70% ethanol, washed with PBS and the chorions were torn with fine forceps. Then the cell mass was released and the chorion membrane were removed. Single cells were obtained by gentle pipetting. After several washes, the cells were transferred into gelatine coated twelve-well tissue plates and after two or three passages (*passage = every passing step from one flask to a new one, to allow cells proliferating*) to six well plates. Cells were incubated at 26°C in a normal atmosphere incubator, according to previous experience with other seabream cell cultures (Béjar *et al.,* 1997). Subculturing was carried out according to the standard trypsinization method.

Alkaline phosphatase staining

This staining technique is routinely used to monitor the undifferentiated state of mouse stem cells in culture (Wobus *et al.*, 1984) and to characterize fish totipotent cells. In our case it was carried out according to Hong and Schartl (1996): Cells were washed with PBS, fixed in acetone/methanol (1:1) for 10 min at room temperature, washed in PBS twice and then stained for alkaline phosphatase activity using bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma) as the

substrate. To examine alkaline phosphatase activity in early embryos, the embryos were fixed in 2% paraformaldehyde, 0,2% glutaraldehyde, 0,02% NP-40 and 1x PBS, then washed several times in PBS and then stained with BCIP/NBT.

Chromosome analysis

For the karyotype study, exponentially growing cultures from passage 4-5 were treated according to the protocol of Alvarez *et al.* (1991) to obtain metaphasic chromosomes.

Growth response assays

Cells were seeded in basal medium at a density of 2.5 x 10^4 cells per well in 12-well tissue culture plates (Table 1). Supplements were immediately added at various concentrations, having found that those specified in Table 2, produced the best results. Cell number was determined four days after plating by counting trypsinized cell suspensions in a Coulter counter.

Table 1. Factors affecting growth

Supplements	Cells (x 10^4/well)
Complete medium	12.4
Omission of LIF (Leukaemia Inhibitory Factor)	12.1
Omission of NPSM (Non-essential amino acids, Na-Pyruvate, Na-Selenite and 2-Mercaptoethanol)	11.7
Omission of SEE (Seabream embryo extract)	11.2
Omission of bFGF (basic Fibroblast Growth Factor)	1.08
Omission of bFGF, SEE and LIF	1.1

Cryopreservation methods

Cells from passages 1, 2 and 3 were cryopreserved following the conditions used for seabream fibroblastic cells (Béjar *et al.* 1997) and stored in liquid nitrogen. For cryopreservation cells were suspended in 10% (v/v) dimethyl-sulfoxide (DMSO) (Sigma) in DME/F12 medium with 20% FBS, placed in cryovials and stored in either liquid nitrogen container or a -80°C freezer from which they were thawed successfully.

Table 2. Medium for seabream ES cells

L-15 Leibowitz Medium
Antibiotics(Pen., Strept. and Amp.)
15% FBS (Sigma)
2% L-Glutamine (4mM)
1% Non-essential amino acids (1mM)
1% Na-pyruvate (1mM)
Na-selenite 2nM
2-Mercaptoetanol 50 mM
Leukaemia Inhibitory Factor (LIF) 10 ng/ml
Basic Fibroblast Growth Factor (bFGF) 10 ng/ml
Seabream embryonic extract (SEE) 20 ml/ml (100% = 5 ml/embryo)

Results and discussion

Primary cultures and subcultures

The first challenge was to find out the optimum development time to initiate cultures. After testing development times between 5-8h, the most successful results were obtained from mid-blastula embryos of 6-7 hours postferlilization, containing approximately 1000 cells (Fig. 1). These results agree with those of Hong and Schartl (1996) in medaka and of Collodi *et al.* (1992) in zebrafish.

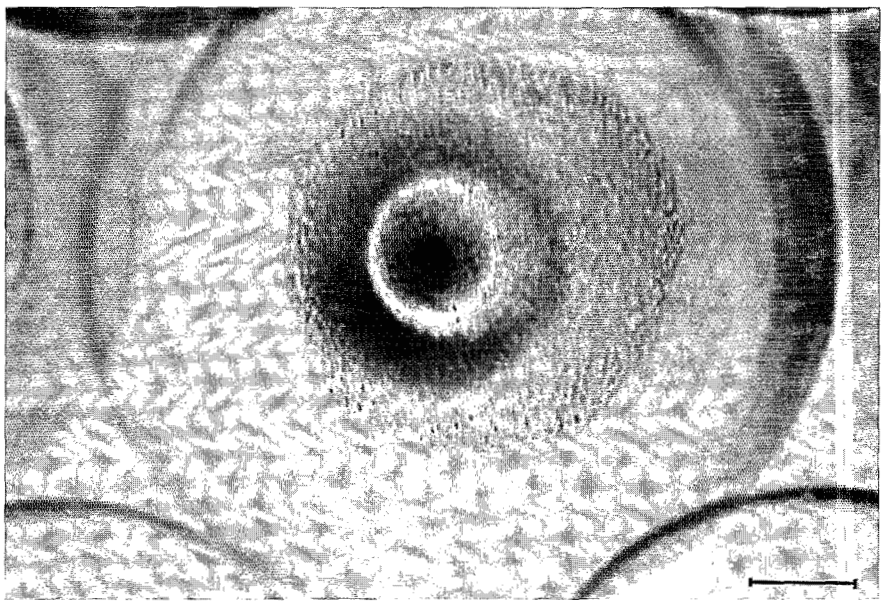

Fig. 1. Midblastula seabream embryo.

Blastomeres show a rounded variable shape and divisions occurred very rapidly during the first hours. Eventually, cells derived from a single blastomere formed an aggregate. Within the first 24h most cells were attached to the well surface and they are very small, round or polygonal in shape and rich in granules (Fig. 2). In general the success of primary cultures mostly depended on a proper initial density and even seeding conditions.

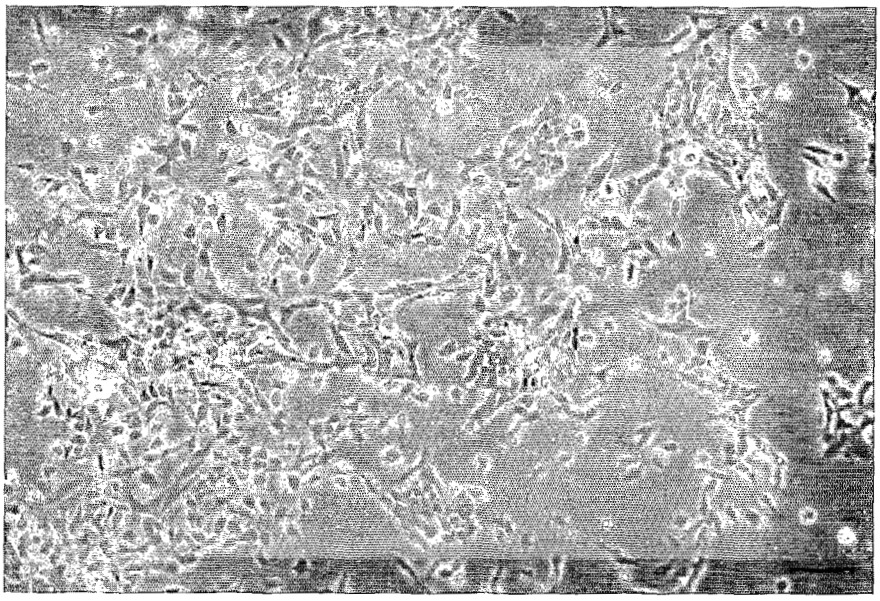

Fig. 2. ES-like cells at passage 2 showing typical morphology of totipotent cells.

After the 2 or 3 passages, many cells started differentiating mostly into fibroblastic-like types, while the remainder retained a morphology similar to that of the primary cultures (Fig. 1). This tendency towards differentiation was highly variable among different cultures, usually increased in parallel to the number of serial passages and was strongly dependent upon culture conditions, especially cell densities. According to our experience, different cell types have been identified by morphological criteria, such as pigmented cells, muscle cells, and neurone-like cells.

The results of cryopreservation tests were very positive, as the mean survival rate after thawing was around 40%, which is quite close to that obtained from a continuous seabream cell line. (Béjar *et al.* 1997). No substantial differences in the survival rate, have been found between cells from the three passages screened.

Medium composition

The medium composition was designed following the indications of Hong *et al.*, (1996) with adaptations relevant to our species. In addition to the general components, various specific supplements for ES-like cells were tested in a dose-dependent way, for their efficiency in growth promotion and for inhibition of cell differentiation. Their relative role in the cell growth is summarised in Table 1, which

indicates that all of them, with the exception of LIF, have an additive effect in stimulating growth, and that bFGF has the greatest effect.

Regarding differentiation, an optimum cell density seems to be crucial for keeping totipotency, which is in turn dependent on the growth rate, as well as on the culture conditions. The cytokine LIF has been acted as a cooperative factor.

The adopted concentrations of each component to produce the optimum complete medium are presented in Table 2. A cell performance evaluation in this medium, based in about 200 primary cultures, revealed that approximately 20% of cells underwent extensive differentiation in passage 1, mostly into fibroblastic-like cells. About 75% differentiated more slowly, reaching in passages 3-4 about 50% of the whole cell population. Beyond that point differentiated cells become dominant. The remaining 5% gave rise to serial ES-like cell cultures that exhibited stable growth and morphology. Cells from passage 6 have been obtained and tested for totipotency.

Tests of totipotency

Alkaline phosphatase staining

This assay was used to compare the staining pattern between, sea bream embryos at mid-blastula stage, which are supposed to be fully totipotent, embryonic primary cultures and putative ES-like cells from passage 6. The results show (Fig. 3) that in mid-blastula embryos all cells showed strong activity, in primary cultures almost all cells exhibited strong alkaline phosphatase activity, while in more advanced passages, only smaller and rounded or polygonal shaped cells are positive, thus indicating that the culture at this stage is the most heterogeneous regarding totipotency.

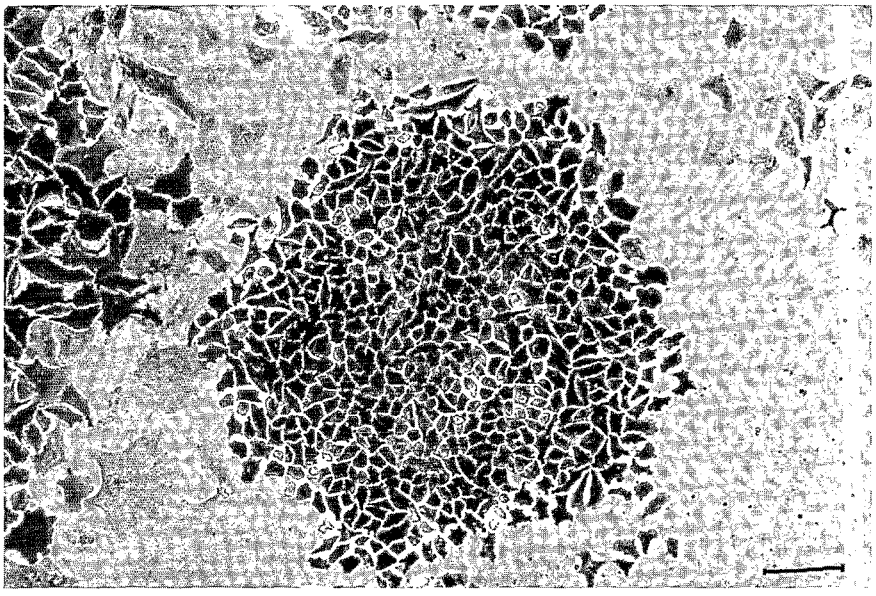

Fig. 3. Alkaline-phosphatase positive cells at passage 6.

Chromosome analysis

In the ES-like cells development process, it is essential to identify lines with an euploid chromosome complement, prior to use in chimaeras production.

The results of the chromosome counts from 21 metaphase plates, obtained from 6 passage cells, reveal a modal chromosome number at 2n=48, with chromosomes showing the standard morphology described for this species (Figs 4 and 5), (Sola and Cataudella, 1978), thus indicating an apparent normal karyotype and an euploid complement. The chromosome values below 48 (Fig. 4), can very likely be explained by artefacts inherent to the technique.

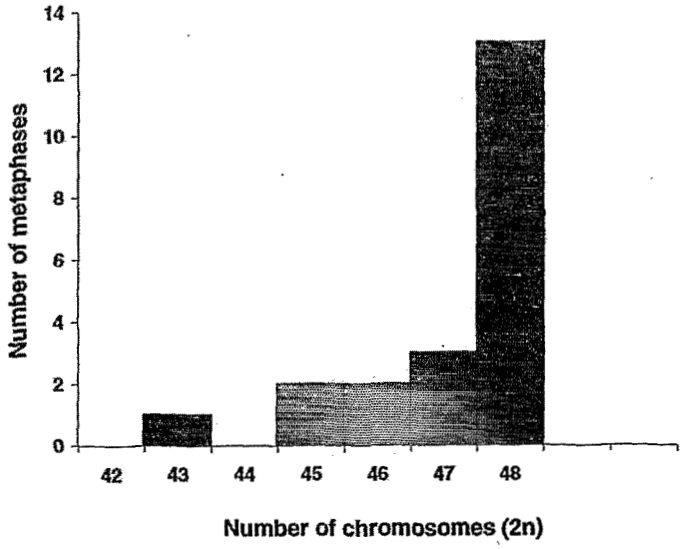

Fig. 4. Distribution of the chromosome number from seabream ES-like cells in passage 6.

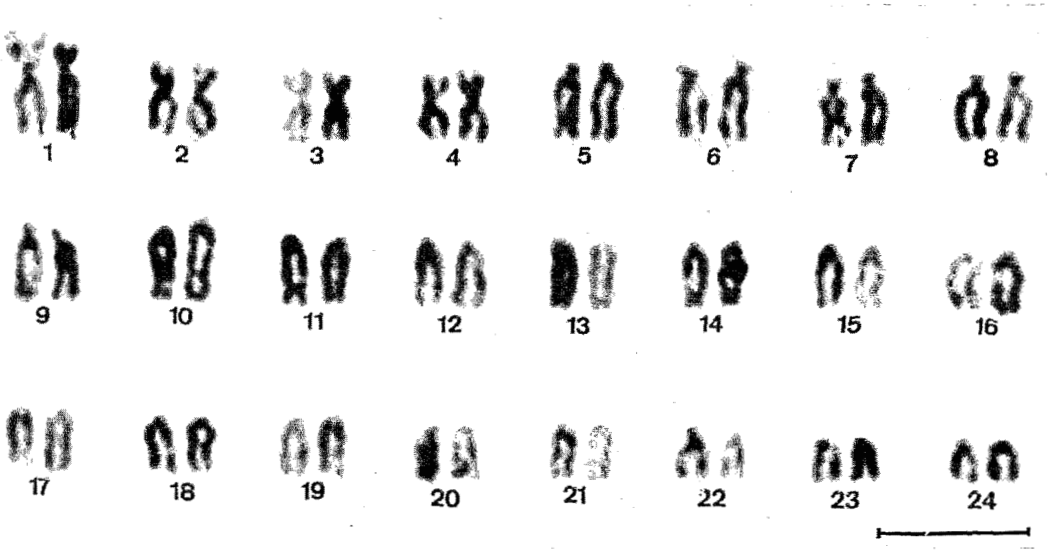

Fig. 5. Karyotipe of the sea bream ES-like cells at passage 6.

This test has to be performed for each ES-like cell line as a quality control, since the chromosome behaviour in cells growing in *in vitro* conditions is unpredictable and can vary among different lines (Ghosh and Chaudhuri, 1984).

Conclusions

In this study the initiation, multipassage cultivation and growth of seabream blastula embryonic cells in the absence of feeder layer cells, is described. The major constraint found was preventing spontaneous differentiation. At present, putative ES-like cells of up to 6 passages are presented. The possible totipotency is suggested by the morphology, the positive alkaline phosphatase staining, the normal karyotype and the ability to differentiate into diverse cell types. However the capacity of these cells to generate embryonic chimaeras has to be demonstrated after being microinjected into suitable recipient embryos. For that purpose, two different approaches are being planned, either the use of albino or haploid embryos. In parallel protocols to transfect ES-like cells with the green fluorescent protein gene and suitable constructions for transformation, are being established.

Acknowledgements

The authors acknowledge the Company Cupimar, S.A. (San Fernando, Cádiz), for providing the eggs and the facilities. This work has been supported by grants BIO2-CT93-0430 from EU and BIO93-1461-CE.

References

Alvarez, M.C., Otis, J., Amores, A. and Guise, K. (1991). Short-term cell culture technique for obtaining chromosomes in marine and freshwater fish. *J. Fish Biol.*, 39: 817-824.

Béjar, J., Borrego, J.J. and Alvarez, M.C. (1997). A continuous cell line from the cultured marine fish gilt-head seabream (*Sparus aurata*). *Aquaculture*, 150: 143-153.

Collodi, P., Kamei, Y., Sharps, A., Weber, D. and Barnes, D. (1992). Fish embryo cell cultures for derivation of stem cells and transgenic chimeras. *Mol. Mar. Biol. Biotech.*, 1 : 257-265.

Ghost, S. and Chaudhuri, A. (1984). Analysis of three whole-arm translocation in a mouse sarcoma cell line. *Cytogenet. Cell Genet.*, 38: 161-164.

Evans, M.J. and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 292: 154-156.

Hong, Y. and Schartl, M. (1996). Establishment and growth responses of early medakafish (*Oryzias latipes*) embryonic cells in feeder layer-free cultures. *Mol.*

Mar. Biol. Biotech., 5 (2): 93-104.

Sola, L. and Cataudella, S. (1978). Prime osservazioni sulla cariologia degli Sparidi Mediterranei. *Boll. Zool.*, 45: 242.

Wakamatsu, Y., Ozato, K., and Sasado, T. (1994). Establishment of a pluripotent cell line derived from a medaka (*Oryzias latipes*) blastula embryo. *Mol. Mar. Biol. Biotechnol.*, 3 (4): 185-191.

Wobus, A.M.,Holzhausen, H., Jakel, P., and Schoneich, J. (1984). Characterization of a pluripotent stem cell line derived from a mouse embryo. *Exp. Cell Res.*, 152: 212-219.