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ORIGINAL RESEARCH

Development of cell line from the testicular tissues of crab *Scylla serrata*

Anumol Shashikumar · P. V. Desai

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Abstract This is the first report on development of a finite cell line from testicular tissues of crab, *Scylla serrata*. Both the explant and segregated tissues of testes yielded cells that could proliferate and grow. These cells ranged in size from 10 to 38 μm with distinct nuclei of varying shapes. The testicular cells survived and proliferated best in L-15-crab saline medium supplemented with epidermal growth factor (20 ng/mL) and glucose (1 mg/mL). The cell proliferation rate was assessed by Methyl tetrazolium assay in terms of change in optical density which clearly indicated a prominent increase in cell density. The testicular cells were subcultured at an interval of 4–6 days. These subcultured cells remained healthy and proliferated for 5 months with a minimum of ten subsequent passages. The finite cell line was characterized in terms of morphology, growth rate, lactate dehydrogenase release (for detecting health status) and 18S rRNA sequencing. This cell line could be a very useful tool for testing infections and replications of crustacean viruses. The present work provides a technique that could be extended for developing other crustacean cell lines.

A. Shashikumar · P. V. Desai (✉)
Department of Zoology, Goa University, Taleigao Plateau, Goa 403206, India
e-mail: pvdesai@unigoa.ac.in

A. Shashikumar
e-mail: anumolkumar@yahoo.co.in

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Abbreviations

EGF	Epidermal growth factor
FBS	Fetal bovine serum
HS	Horse serum
MTT	Methyl tetrazolium
LDH	Lactate dehydrogenase
PCN	Product code number

Introduction

Crustacean cell culture has gained interest over the last two decades, especially when disease problems began to affect the commercially valuable species (Chen et al. 1986; Leudeman and Lightner 1992; Mulford and Austin 1998). Cell culture techniques offer opportunity for studying the effects of pathogens in in vitro state and to develop diagnostic reagents and probes. The availability of such cellular tool is especially important to aquaculture industries which experience disease problems that are exaggerated by intensive aquaculture methods (Ellender et al. 1992; Toullec et al. 1996).

Although primary cell cultures from crustacea have been initiated since 1960s, no established cell line of marine crustacean is reported (Claydon and Owens 2008) except a transformed shrimp lymphoid

cell line using pSV-3 neo vector containing the tumor antigen gene from Simian virus-40 (Tapay et al. 1995) and hybridized *Penaeus monodon* cell line by cellular fusion (Claydon et al. 2010). However, primary cell cultures from several crustacean species using various culture conditions have been reported with increased frequency. Much research pertaining to cultures involved the adjustment of the culture media via nutritional supplementation in an effort to enhance continual mitosis. We have earlier reported primary cell culture of hepatopancreas of *Scylla serrata* and modulation of the culture conditions to enhance survivability of primary cell cultures (Sashikumar and Desai 2008). Though in most cases the cultures survived well, their rate of cell multiplication was low and on subculture no cell proliferation and success beyond the primary stage had been obtained (Toullec et al. 1996). Since there is no established cell line of crustacean species available yet, an attempt was made to develop a cell line from crab *Scylla serrata*, a species of great commercial importance. Such cell lines are essential for isolation, cultivation of crustacean viruses, besides they increase reproducibility of biological investigation of pathogenesis. Therefore, we report here the technique for establishment of a cell line of *Scylla serrata* using testicular tissues.

Materials and methods

Animals

Live male *Scylla Serrata* weighing approximately 110 ± 0.60 g with carapace length of 8–10 cm were obtained from local supplier and were maintained in aerated seawater at salinity 29‰ and temperature 28 °C. Prior to the experiment the crabs were anesthetized by exposing them to ice cold water for 15 min (Merlo and Romano 2007) and were surface sterilized using 10% sodium hypochlorite, followed by swabbing with 70% alcohol.

Culture conditions

Under aseptic conditions the animals were dissected and the testes along with anterior vas deference (AVD) were removed carefully using sterile forceps, avoiding contamination with the gut as well as midgut gland and were transferred to a beaker containing artificial sea water having 0.5% of antibiotic solution (Himedia, PCN: A007-5× 50 mL). The osmolality was adjusted to 1,050 mOsm/kg. After the final wash and further incubation for 5 min in antibiotic solution, the testicular tissues were cut into small fragments of about 1^3 mm.

Explant technique

Explant culture was initiated by seeding finely chopped testicular tissue fragments in 12 well plates, each well contained 2 mL of culture medium. The multi-well plates were incubated at 20–24 °C until a confluent monolayer was formed. Subsequently the tissue fragments were removed and the monolayer was replenished with fresh plating medium having amphotericine-B (100 units), nystatin (100 units) and gentamicin (10 mcg).

Disaggregation technique

The tissue fragments were disaggregated in an artificial sea water (osmolality = 1,050 mOsm/kg). Briefly, the tissue fragments were passed 20 times through a 10 mL pipette (pipette tip 3 mm and wt—35 g) to dissociate cells. The dissociated cell suspension thus obtained was filtered through a nylon mesh (100 μm). The viability of dissociated cells was determined by trypan blue dye exclusion technique. The cell suspension having more than 95% viable cells was used for inoculation at a density of 2×10^5 cells/mL. Disaggregation technique was preferred over explant technique because a known number of cells could be inoculated for culture.

All the cultures were incubated at 20–24 °C and the media were replenished at 2 days interval. Cultured cells were examined daily with an Olympus inverted microscope to observe growth and proliferation. The first subculture was done after 4–6 days of initial inoculation. For subculture, the growing cells were separated by treating them for 1 h with 1% accutase (Sigma, PCN: A6964).

Culture media

The efficacy of various culture media such as Nutrient mixture F-12 (Himedia, PCN: AT024), Leibovitz's (Himedia, PCN: AT011, ×, 2× & 3×)

in combination with Crab Saline (NaCl 440 mM, KCl 11.3 mM, $CaCl_2$ 13.3 mM, $MgCl_2$ 26 mM, Na_2SO_4 23 mM, HEPES 10 mM), Artificial Sea Water (NaCl 480 mM, KCl 10 mM, $MgSO_4 \cdot 7H_2O$ 30 mM, $MgCl_2 \cdot 6H_2O$ 20 mM, HEPES 5 mM), were tested for cell viability, growth and proliferation (Table 1a). The medium supplements such as glucose (1 g/L), epidermal growth factor (10–20 ng/mL, Sigma, PCN: E4127), insulin (0.06 IU/mL), transferrin (5 μM, Himedia, PCN: Rm5475) were used. The fetal bovine serum (Himedia, PCN: 07-359), horse serum (Himedia, PCN: 07-381), heat inactivated sera (30 min at 56° C) of different concentrations (v/v—5, 10, 20%) were used as additional supplements. The osmolality of the media was adjusted to 1,050 mOsm/kg using artificial sea water and Osmometer (Model 3320).

MTT (methyl tetrazolium) assay

Cell proliferation and viability was assessed by MTT test (Mosmann 1983). The assay is based on the intrinsic ability of mitochondrial dehydrogenases of viable cells to cleave MTT (3-[4,5-dimethylthiazol-2-yl]-2 diphenyltetrazolium bromide) to a purple coloured formazan, which is detected spectrophotometrically at 570 nm wavelength after dissolution with MTT solubilisation solution (Sigma, PCN: M8910). Approximately 2×10^5 cells/mL were inoculated in

Table 1 Different culture media and supplements for survivability and proliferation of testicular cells of crab *Scylla serrata*

a	Culture medium tested for cell survivability						
Sr. no.	Medium	Diluents	Osmolality (mOsm/kg)	Substratum			Maximal day of survival
				A	B	C	
1	1× Leibovitz's	SW	1,050	+++	+++	+++	10–25 days
2	2× Leibovitz's	SW	1,050	+++	+++	+++	<75 days
3	3× Leibovitz's	MQW	902	+++	+++	+++	<90 days
4	Leibovitz's + crab saline (1:1)	MQW	1,082	+++	+++	+++	<150 days
5	3× Leibovitz's + nutrient mixture F12 (HAM) + SW (1:1:1)	MQW	1,088	+++	+++	+++	<123 days
6	F-12 (HAM)	SW	1,050	+++	+++	+++	<90 days
7	Sea water + glucose	MQW	1,050	+++	+++	+++	15–30 days

b	Medium supplements with growth factors	
	Cells culture tested for proliferation using MTT assay after 48 h of initiation of culture	OD
1	L15 + CS + glucose (1 g/L) (control culture medium).	0.30 ± 0.0024
2	L15 + CS + EGF (10 ng/mL) + glucose (1 g/L)	0.34 ± 0.0005
3	L15 + CS + EGF (20 ng/mL) + glucose (1 g/L)	0.43 ± 0.0037
4	L15 + CS + insulin (0.06 IU/mL) + transferrin (5 μM) + glucose (1 g/L)	0.31 ± 0.0011
5	L15 + CS + 0.06 IU/mL insulin + transferrin (5 μM) + EGF (20 ng/mL) + glucose (1 g/L)	0.39 ± 0.0003

c	Chemically defined medium supplemented with sera	
	Cells tested for proliferation using MTT assay after 48 h of initiation of culture	OD
1	L15 + CS + EGF 20 ng/mL + glucose (1 g/L) (control culture medium)	0.68 ± 0.0010
2	L15 + CS + EGF 20 ng/mL + glucose (1 g/L) + horse serum (5%)	0.43 ± 0.0045
3	L15 + CS + EGF 20 ng/mL + glucose (1 g/L) + heat inactivated horse serum (5%)	0.40 ± 0.0050
4	L15 + CS + EGF 20 ng/mL + glucose (1 g/L) + fetal bovine serum (5%)	0.37 ± 0.0015
5	L15 + CS + EGF 20 ng/mL + glucose (1 g/L) + heat inactivated fetal bovine serum (5%)	0.29 ± 0.0045

A collagen coated petri dishes, *B* poly D lysin coated petri dishes, *C* laminin coated petri dishes, *MQW* mili Q water, *SW* seawater, +++ approximately 90–95% of testicular cells adhere on the petri dish surface, *CS* crab saline, *OD* indicates activity of mitochondrial dehydrogenase of live cells. Dead cells do no show +ve OD for MTT assay. Increase in OD indicates cell proliferation

culture plates having medium supplemented with growth factors (Table 1b, c) and the cells were monitored for viability and proliferation. MTT assay was conducted according to the manufacturer's instructions (Sigma, PCN: TOX1).

Mitotic index

The percentage of cells in mitosis was determined by counting mitotic cells in a culture as a proportion of the whole population. The cell cultures were stained with aceto-carmine.

LDH (lactate dehydrogenase) assay

Lactate dehydrogenase leakage was estimated by measuring LDH activities in culture medium and cell lysate using LDH-in vitro toxicity assay kit (Sigma, PCN: TOX 7). Cultured cells were lysed using lyses solution (of the LDH assay (Sigma, PCN: L2152)), and the cell lysate after centrifugation at 5,000 rpm for 10 min was used as the non-damaged control. Culture medium was centrifuged at 5,000 rpm for 10 min at 4° in order to remove cells or cell debris. The obtained supernatant and cell lysate was mixed with working solution from the kit. The mixture was stirred and incubated at room temperature for 30 min. The reaction was stopped by adding 1N HCl solution. Absorption of the reaction mixture was measured at 490 nm using Nanodrop (ND-1000) spectrophotometer. LDH leakage was calculated using the following formula: LDH leakage $(\%) = 100 \times (CS - BC)/(NDC - BC + CS - BC)$. CS, BC and NDC represent absorption of culture supernatant, background control and non-damaged control, respectively.

18S rRNA sequencing

The cultured cells and parental tissue genomic DNA was isolated using GenElute Mammalian Genomic DNA kit (Sigma–Aldrich G1N10) following the manufacturer's recommendation. The DNA extracts were stored at −20 °C until used. PCR amplification was carried out following the method of Williams and Ozawa (2006). The 18S rRNA gene was amplified using primers 18S-5_(F) and 18S1100R (R), the sequences of forward and reverse primers were 5′-CTG GTT GAT YCT GCC AGT-3′, 5′-CTT CGA ACC TCT GAC TTT CG-3′. Amplification reaction was carried out in 50 µL of reaction mixture that consisted of 2× PCR master mix (26 µL, Sigma-P 4600), Forward primer 100 pM/µL (2 µL), Reverse primer 100 pM/µL (2 µL), Template DNA 80 ng/µL (1 µL), PCR water (19 µL). The PCR reaction was initiated by heating the mixture for 5 min at 94 °C followed by 30 cycles, 1 min at 94 °C, 1 min at 54 °C, 2 min at 72 °C and 7 min at 72 °C with a final extension till end point at 4 °C. The resulting amplified products (10 µL) were mixed with 2 µL loading dye (6X DNA loading Dye of Fermentas #R0611) and loaded onto 1.2% agarose gels, subsequently stained with ethidium bromide and viewed under UV-light. All the PCR products were sequenced at MWG Biotech, Bangalore, India and chromatograms were obtained. The sequences of PCR products were analyzed by using Basic Local Alignment Search Tool (BLAST). The sequence was deposited in Gene bank, [National Center for Biotechnology Information, (NCBI)] Maryland, USA.

Result

Development of primary culture from testicular tissue

Testicular tissues were cultured successfully from explant. A rapid migration of spermatogonial cells occurred from the explant tissues within a few hours of initiation of culture leading to the formation of about 70% of cell confluence by the end of 24 h.

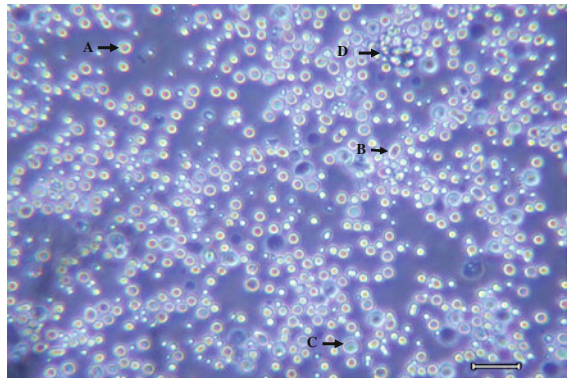

Fig. 1 Cell culture from testicular tissues of crab, *Scylla serrata*. *A* Round cells of ~10–18 µm, *B* cell of ~15–28 µm, *C* large cells of ~21–38 µm, *D* detached cell clumps. All the cells are spread and attached to the substratum except the clumps. *Scale bar* 60 µm

Cells ranging from ~10 to 18, 15 to 28 and 21 to 38 μm in size with nuclei varying in size and shape (Fig. 1) migrated from the explant. Cell size and shape was determined with Image analyzer (Nikon Eclipse TS100, TI-SM Japan). By contrast, the disaggregation technique resulted in a heterogeneous population of cells ranging from ~10 to 38 μm in size. Some of the cells detached and had the tendency to form clumps (Fig. 1).

Effect of different media and supplements on cell proliferation

Different media combinations and supplements (Epidermal growth factor—EGF, transferrin and Insulin) were tested to find a suitable medium formulation that can promote proliferation and longevity of primary cultures. L15 medium prepared in crab saline with osmolality of 1,050 mOsm/kg promoted survivability of <150 days (Table 1a), and therefore it was used for subsequent studies for testing cell proliferation using supplements. We varied the concentrations of EGF, transferrin and insulin in the media to examine cell proliferation using the MTT assay. L15—crab saline supplemented with EGF (20 ng/mL) and glucose (1 g/L) facilitated maximum proliferation of cultures (Table 1b). With insulin, transferrin supplements the proliferation of cells was poor. The chemically defined media (CDM = L15 + crab saline supplemented with EGF 20 ng/mL, glucose 1 g/L) was then supplemented with different concentrations of sera for testing proliferation.

When different concentrations of sera were tested, 5% (v/v) seemed to support proliferation best. Therefore, CDM and 5% serum combination was used for evaluating the rate of proliferation and longevity of the cultures. Among the sera supplements, 5% horse serum yielded better results (Table 1c). However, MTT assay revealed that plain CDM was most suitable for cell proliferation and longevity in comparison with sera supplements (Table 1c).

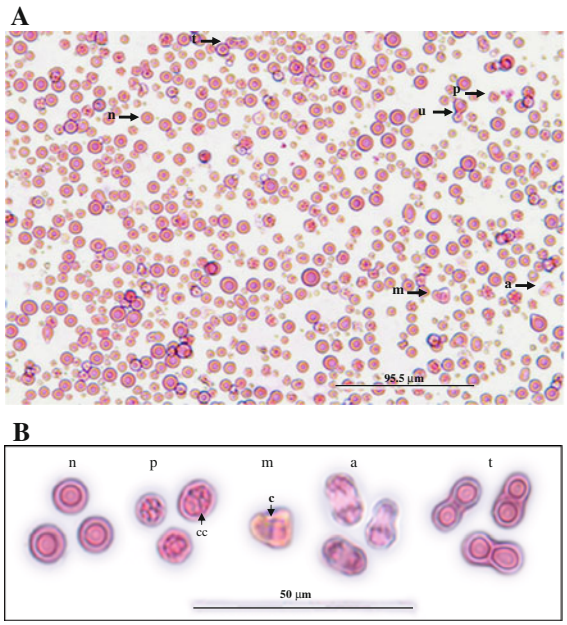

Fig. 2 **a** Testicular cell culture of *Scylla serrata* stained with aceto-carmine. *n* non dividing mature cells, *p* prophase, *m* metaphase, *a* anaphase, *t* telophase, *u* unequal division. **b** Magnified mitotic stages picked up from **a** as well as from other slide preparations for clarity. *cc* condensed chromosomes, *c* chromosomes at equatorial plate

Subculture of testicular cells

The first subculture was initiated at an interval of 4–6 days (of primary culture) at a requisite density of 2×10^5 cells/mL in a chemically defined medium for developing a cell line. Subsequent subcultures were performed at an interval of 2 weeks and the cells appeared healthy and proliferating. These subcultures survived ten subsequent passages and remained healthy and proliferated for more than 5 months. The cells cultured using plain culture medium without supplements exhibited mitotic index equivalent to 19, while those cultured using CDM showed mitotic index equal to 26. Even the subcultures/cell lines grown in CDM maintained the same (26) mitotic index. Figure 2a, b clearly shows the presence of non-dividing as well as mitotic cells, especially those in prophase, metaphase, anaphase and telophase stages. The total amount of LDH release of cultured testicular cells was always less than 10% from 24 h of inoculation to the end of 5 months. This result indicates that the cells are healthy, as in any culture conditions less than 10% LDH release is believed as a normal phenomenon.

18S rRNA sequencing

Genetic lineage of the cultured cells from the parental tissue as well as it's relatedness with its genus and other species is assessed by 18S rRNA sequence.

Fig. 3 18S rRNA partial sequences from testicular cell of crab *Scylla serrata*

GGGCTGTTGGTTAGCATGCATGTCTAGTA CAGCCGATTAGGTGAACCGCGAATGG
CTCATTAAATCAGCTATGAT TCATTGGATCTGTAGCCCACA CTTACTTGGATAACTGTGGT
AATTCTAGAGCTAATACATGCACCACGTCT CTGACCGCAAGGGAAGAGCGCTTTTATTAGT
TCAAAACCGGTCGGGCCTCGGTCCGCCAAC CACACCGTGTTGAATCTGAATAACTTGTGG
CTGAGCGCACGGCCTCCAGTGCCGGCGCCGCCT CTTTCAAGTGTCTGCCTTATCAGCTTTC
GATTGTAGGCTATGCGCCTACAATGGCTAA AACGGGGAACGGGGAATCATTTTTTTAAGC
GGGAAAGAAGCATGAGAGGGGGAAAAGGAG GAGGGGGGGGACAGGGGGACCAAAATTG
GCGAACAGA

The testicular cells' sequences showed 425 bases with a base count of 109 adenine, 94 cytosine, 123 guanine, 99 thymine (Fig. 3). The 18S rRNA sequence of testicular cells when analyzed using BLAST showed 98% phylogenetic similarity to *Scylla paramamosain's* 18S ribosomal RNA (gene accession no FJ774907) and 97% similarity to *Scylla paramamosain's* hypothetical protein mRNA (gene accession no FJ774884) indicating that the cultured testicular cells were derived from parental tissue of *Scylla.* The gene bank accession number for our 18S rRNA sequence for *Scylla serrata* is HQ697253. The parental tissues' 18S rRNA sequence matched with cultured cells 18S rRNA sequence.

Discussion

This is the first report of development of a crustacean cell line that could survive for 5 months with 10 passages. The lack of standardized system for growth of long lasting primary cultures which could eventually lead to establishment of a true finite cell line, created an obstacle to study the pathogenesis of crustacean viruses (Chen and Wang 1999; Claydon and Owens 2008). We have earlier reported long surviving hepatopancreatic primary culture of *Scylla serrata* using serum free medium (Sashikumar and Desai 2008). After reporting hepatopancreatic primary culture we looked for other tissues of crab that can develop a finite cell line with a long survivability. Therefore, we used testicular tissues of crab for explants and segregated cell cultures.

Both the explants and segregated tissues of testes yielded cells that could proliferate and grow. These cells ranged in size from 10 to 38 μm with distinct nuclei of varying shapes. The testicular cells on exposure to different combinations and strengths of L-15 supplemented with growth factors such as EGF, insulin, transferrin, glucose and different sera showed varying survivability and proliferation rates. The use of L15 as a suitable culture medium for crustaceans has been demonstrated by many researchers (Nadala et al. 1993; Hsu et al. 1995; Tong and Miao 1996; Fraser and Hall 1999; Owens and Smith 1999). However, the present study indicates that F-12, L-15 (X, 2X & 3X) are less suitable for proliferation of primary and subcultures of testicular cells while L-15 with crab saline (1:1) can promote 5 months survivability with limited cell proliferation. However, this combination requires additional supplements like EGF (20 ng/mL) and glucose (1 g/L) to promote good proliferation and extended survivability of cells. Nadala et al. (1993) has shown the efficacy of EGF on Oka cell proliferation from *Penaeus stylirostris* and *Penaeus vannamei*. Tapay et al. (1995) reported benefit of 20 ng/mL of EGF in the medium formulation to culture lymphoid cells of *P. Stylirostris*. In the present study we find that the addition of EGF promotes rapid cell proliferation (mitotic index = 26).

Mulford and Austin (1998) have shown the necessity of serum at a concentration of 20% (v/v) as an ingredient in *Nephrops norvegicus* testis cell culture. However, our results show that with 20% concentrations of different sera caused cell deterioration within a few days while use of 5% (v/v) serum extended cell survivability. Leudeman (1990) and Frerichs (1996) suggested that serum could be toxic for crustacean cells. Moreover Walton and Smith (1999) reported occasional clumping of cultured hemocytes of crabs (*Liocarcinus depurator* and *Carcinus maenas)* in 20% (v/v) serum, while 10% serum significantly promoted the viability of culture. Perhaps this could explain why the higher level of sera i.e. 15–20% resulted in a shorter period of survival for testicular cells than that observed in five percent. Addition of horse serum in culture medium promoted relatively better cell proliferation and

survivability than that promoted by fetal bovine serum supplement.

The very first subculture from the primary cell culture is a cell line (Freshney 2000). Therefore, the subcultures of ten passages with proliferation and survivability for 5 months could definitely be considered as finite cell lines of testicular tissues of *Scylla serrata*. The cell proliferation and growth exhibited by the testicular cells only in presence of epidermal growth factor (EGF) suggests that they are epithelial types. The unequal mitotic cell division observed in a few testicular cells of crab gives an impression of budding yeast. However, there is no possibility of presence of budding yeast in cell culture as the culture medium contained antibiotics like amphotericin-B and nystatin which are anti-yeast and anti-fungi. Since crab testicular cells have 53 haploid chromosomes, they are not quite prominently seen in chromosomal preparations, but the mitotic stages are clearly indicated by distinctly condensed chromosomes in prophase, equatorially arranged chromosomes in metaphase and their polar alignment in anaphase. Telophase is characterized by two distinct nuclei with partial cell separation.

Recently, Claydon et al. (2010) have attempted developing a hybridized *Penaeus monodon* cell line by cellular fusion for testing virus pathogenicity but it is reported that such cells lack crustacean components required for the use as an in vitro system for virus replication. Therefore, this testicular cell line could be a very useful tool for testing infection and replication of crustacean viruses such as WSSV, hypodermal and haematopoietic necrosis viruses that cause damages to the aquaculture industry. Nevertheless, the present study provides a technique that could be extended for developing other crustacean cell lines.

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References

Chen SN, Wang CS (1999) Establishment of cell culture systems from penaeid shrimp and their susceptibility to white spot disease and yellow head viruses. Methods Cell Sci 21:199–206

Chen SN, Chi SC, Kou GH, Liao IC (1986) Cell cultures from tissues of Grass Prawn, *Penaeus monodon*. Fish Pathol 21:161–166

Claydon K, Owens L (2008) Attempt at immortalization of crustacean primary cell cultures using human cancer genes. In Vitro Cell Dev Biol Anim 44:451–457

Claydon K, Roper GK, Owens L (2010) Attempt at producing a hybridised *Penaeus monodon* cell line by cellular fusion. Fish Shell fish Immunol 29:539–543

Ellender RD, Najafabadi AK, Middlebrooks BL (1992) Observation on the primary culture of hemocytes of Penaeus. J Crustac Biol 2(2):178–185

Fraser CA, Hall MJ (1999) Studies on primary cell cultures derived from ovarian tissue of *Penaeus monodon*. Methods Cell Sci 21:213–218

Frerichs GN (1996) In vitro culture of embryonic cells from the freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 143:227–232

Freshney RI (2000) Culture of animal cells. In: John W (ed) A manual of basic techniques, 4th edn. Inc publication, New York, pp 177–193

Hsu YL, Yang YH, Chen YC, Tung MC, Wu JL, Engleking MH, Leong JC (1995) Development of an in vitro subculture system for the Oka organ (lymphoid tissue) of *Penaeus monodon*. Aquaculture 36:43–55

Leudeman RA (1990) Development of in vitro primary cell cultures from the penaeid shrimp *Penaeus stylirostris* and *Penaeus vannamei* and evaluation of a potential application. Master Thesis, The University of Arizona, Tucson, AZ

Leudeman RA, Lightner DV (1992) Development of an in vitro primary cell culture from the penaeid shrimp *Penaeus stylirostris* and *Penaeus vannamei*. Aquaculture 101:205–211

Merlo E, Romano A (2007) Long-term memory consolidation depends on proteasome activity in the crab *Chasmagnathus*. Neuroscience 147:46–52

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Mulford AL, Austin B (1998) Development of primary cell cultures from *Nephrops norvegicus*. Methods Cell Sci 19:269–275. doi:10.1023/A:1009787223797

Nadala ECB, Lu Y, Loh PC (1993) Primary culture of lymphoid, nerve, and ovary cells from *Penaeus stylirostris and Penaeus vannamei*. In Vitro Cell Dev Biol Anim 29A:620–622

Owens L, Smith J (1999) Early attempts at production of prawn cell lines. Methods Cell Sci 21:207–211

Sashikumar A, Desai PV (2008) Development of primary cell culture from *Scylla serrata*: primary cell cultures from *Scylla serrata*. Cytotechnology 56:161–169

Tapay LM, Lu Y, Brock JA, JR NadalaECB, Loh PC (1995) Transformation of primary culture of shrimp (*Penaeus stylirostris*) lymphoid (Oka) organ with simian virus- 40 (T) antigen. Proc Soc Exp Biol Med 209:73–78

Tong SI, Miao HZ (1996) Attempts to initiate cell cultures from *Penaeus chinensis* tissues. Aquaculture 147:151–157

Toullec JY, Crozat Y, Patrois J, Porcheron P (1996) Development of primary cell cultures from the penaeid shrimps

Penaeus vannamei and *P. indicus*. J Crustacean Biol 16:643–649. doi:10.2307/1549183

Walton A, Smith VJ (1999) Primary culture of the hyaline haemocytes from marine decapods. Fish Shellfish Immunol 9:181–194

Williams ST, Ozawa T (2006) Molecular phylogeny suggests polyphyly of both the turban shells (family Turbinidae) and thsuperfamily Trochoidea (Mollusca: Vetigastropoda). Mol Phylogenet Evol 39:33–51