Unveiling the molecular and regulatory mechanisms that prevent in vitro transformation in shrimp remains elusive in the development of continuous cell lines, with an arduous history of over 25 years (Jayesh et al., 2012). Despite presenting challenges to researchers in developing a cell line, the billion dollar aquaculture industry is under viral threat. In addition, the regulatory mechanisms that prevent in vitro transformation and carcinoma in shrimps might provide new leads for the development of anti-ageing and anti-cancer interventions in human (Vogt, 2011) and in higher vertebrates. This highlights the importance of developing shrimp cell lines, to bring out effective prophylactics against shrimp viruses and for understanding the mechanism that induce cancer and ageing in human.

Advances in molecular biology and various gene transfer technologies for immortalization of cells have resulted in the development of hundreds of cell lines from insects and mammals, but yet not a single cell line has been developed from shrimp and other marine invertebrates. With this backdrop, the research described in this thesis attempted to develop molecular tools for induced in vitro transformation in lymphoid cells from Penaeus monodon and for the development of continuous cell lines using conventional and novel technologies to address the problems at cellular and molecular level.

The first chapter of the thesis has dealt with perception and orientation of the efforts made worldwide in establishing cell lines from shrimp and explained the importance of developing lymphoid cell line from P. monodon. Subsequently, the development of a novel medium for lymphoid cell culture and the impacts of this
medium on lymphoid cells at cellular and molecular level have been addressed in the second and third chapters. These results highlighted the importance of lymphoid cell culture being selected for testing the telomerase activity (Chapter fourth) and found that the spontaneous immortalization of lymphoid cell in vitro would not be possible due to inadequate expression of telomerase; despite telomerase reverse transcriptase gene (\textit{PmTERT}) has been identified from \textit{P. monodon}. This led to the research intended on oncogene transduction and induction of in vitro transformation in lymphoid cells using recombinant baculovirus with hybrid promoter system, which were explained in the subsequent chapters (Chapter fifth and sixth). With this background, the thesis was conceptualized and executed focusing on molecular approaches for the development of cell lines from lymphoid organ of \textit{P. monodon} using most recent techniques. However, continuous cell lines have not yet been achieved through this research, despite the generation of ‘stable’ lymphoid cell culture and the standardization of molecular tools for in vitro transformation with a future perspective.

The subject matter in this thesis has been divided under the following heads:

1. A novel medium for the development of in vitro cell culture system from \textit{Penaeus monodon}
2. Screening and optimization of growth factors and their potential impacts on lymphoid cell culture: Cellular activity and viral susceptibility
3. Differential expression of telomerase in various tissues and primary lymphoid cell culture, and identification and partial sequencing of telomerase reverse transcriptase (\textit{TERT}) gene in \textit{Penaeus monodon}
4. Construction and evaluation of the versatile recombinant baculoviral vector systems with hybrid promoters designed for the expression of foreign gene in shrimp cells
5. Transfection and transduction mediated oncogene expression in lymphoid cell cultures from *Penaeus monodon* for its *in vitro* transformation

**Overall achievements of this work are summarized as given below:**

- Haemolymph composition of *P. monodon* including 16 amino acids, 10 metal ions and 15 fatty acids were quantified, hitherto not recorded in this species.

- Amino acid components in haemolymph could be determined, and they were aspartic acid (17.24%), threonine (4.6%), serine (7.53%), glutamic acid (12.2%), proline (1.3%), glycine (7.55%), alanine (6.12%), cystine (0.18%), valine (8.35%), isoleucine (5.17%), leucine (9.56%), tyrosine (2.32%), phenyl alanine (7.69%), histidine (6.84%), lysine (1.18%), and arginine (0.85%).

- Haemolymph metal ions and the metal ion composition in seawater (27‰) were analyzed and compared. In haemolymph, the metal ions such as sodium (6784.3±785.8 mg l⁻¹), potassium (524.5±157.9 mg l⁻¹) and calcium (488.8±107.9 mg l⁻¹) were found to be within the range of that of 27‰ seawater which contains 8075.5±260.9 mg l⁻¹, 512.935±73.2 mg l⁻¹ and 443.7±63.1 mg l⁻¹ of the metal ions respectively.

- It was identified that, out of the 15 fatty acid in haemolymph, 81.63% were found to be contributed by palmitic acid (16:0), linoleic acid (18.2 ω-6), oleic acid (18.1 ω-9) and stearic acid (18:0). Other fatty acids recorded were capric acid (0.07%) lauric acid (0.17%), myristic acid (1.28%), pentadecyclic acid (0.48%), margaric acid (1.95%), linolenic acid (0.16%), nonadecyclic acid (0.53%), arachidic acid (0.46%), eicosenoic acid (0.34%), eicosadienoic acid (0.34%) and arachidonic acid (2.58%).
A novel cell culture medium (seawater based) has been designed and formulated based on haemolymph composition and designated as shrimp cell culture medium (SCCM). Techniques were developed for generating primary cell cultures from the tissue/cells/organ of *P. monodon* using this medium.

In SCCM, the lymphoid cell culture could be maintained for 85±9 days during which they showed better proliferation among all the cell types tested and exhibited an increase of 107% growth in comparison with 2x L-15, and 59% and 82% in comparison with modified L-15 and Grace’s insect medium respectively (*p* <0.05).

Primary cell culture could be generated from most of the organs/tissues and the cell longevity was 63±6 days for cells derived from ovary followed by that of heart (29±1 days) hepatopancreas (25±5 days) testis 21±3 days, haemocytes 10±3 days, eye stalk 9±2 days, muscle 7±1 days, nerve cord and cells from nauplii 6±1 days.

The experiments by addition of organic supplements like vitamin mixture, lipid mixture, citric acid cycle intermediates, nitrogenous base and energy precursors over and above incorporated in the basal medium did not bring forth any enhancement in the attachment of cells, their proliferation and confluence.

Shrimp cell dissociation ‘cocktail’ was designed for passaging the primary cell culture and with this, better survival (40%) of lymphoid cells was observed even after two passages.

Screening and optimization of growth factors were performed to select most suitable growth factor for lymphoid cells *in vitro* using Plackett-Burman and Central Composite Design of Response Surface Methodology,
which were hitherto not attempted to screen and optimize growth factors in
the media for shrimp cell culture.

Insulin growth factor –I and II (IGF-I and IGF-II) were found to be
effective for the development and maintenance of lymphoid cell culture
with a concentration 100 ng ml\(^{-1}\) and 150 ng ml\(^{-1}\) respectively.

IGF-I and IGF-II induced increase of 24.8% in BrdU incorporation, 0.84%
in protein synthesis, 16.5% in mitochondrial dehydrogenase activity and
17.5% in glucose assimilation within 48 h of incubation. Moreover, the
glucose assimilation rate was elevated to 53.6% (p <0.05) within 72 h of
incubation.

Immunofluorescence detection of synthesis phase in lymphoid cells was
performed, and found that 24±2% of the cells were in S-phase after 48 h of
incubation in SCCM.

Cell cycle gene expression profile was determined and found that 19.7%
increase in the expression of gene encoding transcription elongation factor
in lymphoid cells \textit{in vitro}, while comparing with its tissue counterpart.

Studies on organization of F-actin filaments were performed and the
petaloid nature of F-actin filaments was observed in the lymphoid organ
cells grown in SCCM.

Mitotic events in lymphoid cells were recorded and confirmed that the
cells were mitotically active. This was achieved by time-lapse imaging.

The lymphoid cell culture was found to be susceptible to WSSV and as
cytopathic effect shrinkage, rounding, and detachment of infected cells was
the common features in the event. This was further confirmed by
immunofluorescence detection of viral protein employing monoclonal
antibodies (MAb-C38) against WSSV.
Telomeric repeat amplification protocol (TRAP) for measuring the telomerase activity in various tissue/organ/primary culture from *penaeus monodon* was standardized.

Telomerase activity in the primary lymphoid cell culture was found to be inadequate for maintaining telomere repeats at the chromosome ends during continuous cell division.

Internal amplification standard (ITAS) as internal control for TRAP assay was designed using *P. aeruginosa* MCCB 103 16S rRNA gene sequence along with TS and CX-ext primers.

TTAGG repeats were found to be the canonical telomeric repeats of *P. monodon*.

Identification and partial sequencing of telomerase reverse transcriptase gene (*PmTERT*) from *P. monodon* was performed and found 100% sequence similarity with *Daphnia pulex TERT* gene.

Green fluorescent protein expressing recombinant baculovirus was constructed and expressed in insect cell line (Sf9).

Two recombinant baculovirus transduction vectors (BacLe1-GFP and BacP2-GFP) that carried expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Le1 or PH-P2 was successfully constructed and expressed in insect cell line and shrimp cells *in vivo* and *in vitro*.

The method of virus transduction in lymphoid cells was standardized using recombinant baculovirus.

Optimized the condition for lipofection mediated gene transfer in lymphoid organ cells using Cellfectin (Invitrogen) and the positive signals of green
fluorescence from the reporter green fluorescence protein (GFP) in pEGFP-C1 vector indicated the transfection efficiency.

✓ Transfection of SV40-T oncogene in to primary lymphoid organ cells proved the immortalization potency. The transformed cells showed abnormal nucleus and activated mitotic division.

✓ Recombinant baculovirus expressing 12S E1A oncogene tagged with GFP was successfully transduced in to lymphoid cell culture and the successful transformation was confirmed by protein expression and GFP expression from the transduced cells.

**Scope for future research: following have been identified for future research**

- Oncogene induced up-regulation of cell cycle gene (s) in cells *in vitro*.

- Application of RACE (Rapid Amplification of cDNA Ends) in characterization of *PmTERT* gene from *P. monodon*.

- Telomerase activation in lymphoid cells by ectopic expression of *PmTERT* gene.

- Impact on co-transfection and expression of oncogene and telomerase reverse transcriptase (*TERT*) gene in cells *in vitro*.

- Epigenetic control in shrimp cells that prevent spontaneous *in vitro* transformation.

- Development of fusion (hybrid) cell lines from *P. monodon*.

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