Study on the establishment of continuous cell culture from Kuruma shrimp Marsupenaeus japonicus

[課程博士・論文博士共通]

博士学位論文内容要旨
Abstract

専　攻 Major	Applied Marine Bioscience	氏　名 Name	Thitiporn Thammasorn
論文題目 Title	Study on the establishment of continuous cell culture from Kuruma shrimp, *Marsupenaeus japonicus*		

Development of shrimp cell culture offers opportunities for studying the cellular and molecular level of shrimp pathogens and development of diagnostic tools. The development of shrimp cell culture has been studied over three decades since 1986 with several techniques. However, no continuous shrimp cell lines have yet been successfully established. This study attempts to establish the continuous cell line from kuruma shrimp, *Marsupenaeus japonicus*. This study consists of two parts, investigation and establishment.

To investigate the molecular mechanisms that control shrimp cell proliferation and cellular arrest, shrimp primary cell culture was studied. This study aimed to comprehensively identify key regulator genes that control cell cycle progression in shrimp culture conditions. The primary shrimp cells were derived from shrimp organs and cultured in control condition (*in vitro*). RNA sequencing (RNA-Seq) was performed to investigate gene expression profiling of shrimp primary cells, follows by differentially expressed genes (DEGs) analysis which aims to investigate the changes in gene expression between shrimp primary cells (*in vitro*) and shrimp tissue (*in vivo*). Two research works were performed to provide understanding on the molecular level of shrimp primary cell culture. (i.) the change in gene expression level of shrimp testis primary cells were investigated utilizing DEG analysis. RNA-seq results revealed over 100 genes with distinct gene expression patterns between primary cells and normal tissue. The functional gene analysis of DEG results showed a clear difference in gene expression patterns of cell cycle-related genes between primary cells and normal tissue. LRWD1, TMEM127, CDCA3, PPP2R1A, and GOLGA2, which are cell cycle-related genes, were downregulated in shrimp primary cells. These genes are required for cell proliferation control, G1/S phase transition and entry into mitosis. However, a gene that induces cell cycle arrest, ARAF, was upregulated in culture conditions. The results demonstrated that these genes might play essential roles in cell proliferation and cell arrest under culture conditions. (ii) several shrimp organ types have been used in an attempt to establish shrimp cell line. However, no continuous shrimp cell lines have been successfully established from any cell types. To identify shared DEGs of cell cycle regulator genes, RNA-seq analysis across four types of primary cell including hematopoietic, lymphoid, testis, and ovary were performed. The DEGs revealed over 50 DEGs that were co-expressed among four types of organs' primary cell. Several down-regulated shared DEGs were classified as 'transporters' which involved in transportation of iron, anion, cation, and protein. Among down-regulated genes, 9 genes were necessary in cell proliferation. Up-regulated DEGs were identified as ubiquitin system and promote growth arrest. Moreover, 76 genes from four types of primary cell were found to be associated with cell cycle-related process such as cell cycle regulation, DNA replication, spindle assembly, and mitotic spindle. The PPI network suggested that down-regulated DEGs, DLGAP5, BIRC5, BUB3, and GSTP1 were co-expressed in the same network and interacted with several genes that involved in mitotic process including Cyclin B, CDC20, and PLK1. Furthermore, up-regulated DEGs of cell cycle regulation, NUAK1 was found the be associated with genes that involved in cellular senescence including TP53, BTRC, and FBXW11. These results provide further understanding on molecular mechanism and key molecular pathways under shrimp primary cell that might play essential roles in cell proliferation and cellular arrest. These candidate genes may be an alternative potential target for genetic manipulation to maintain cell proliferation and establishment of a shrimp continuous cell line.

The second part of this project which aims to establishment shrimp continuous cell line, three methods were performed (i) gene silencing via RNA interference, (ii) 3-dimensional (3D) cell culture technology, and (iii) chemical treatment. First, gene silencing via dsRNA targeted two main cell cycle inhibitor genes, RB and p53, in shrimp primary cell. RB and p53 genes are tumor suppressor genes that play roles in the controlling and inhibition of cell cycle progression. Thus, we hypothesized that generation of RB and p53-deficient cell might

promote unlimited cell proliferation led to an establishment of continuous shrimp cell culture. Results from 3 days after transfection showed that RB and p53 mRNA level were successfully suppressed in shrimp primary cell via effect of mixed-dsRNA with 3-times transfection. Interestingly, Cyclin B and Cyclin E expression level were significantly increased in RB and p53-deficient cell. However, mRNA level of RB and p53 showed re-expressed of mRNA level similar to no-dsRNA treated group 6 days after transfection which result in down-regulation of cyclin related genes. These results suggested that gene silencing via dsRNA could down-regulated mRNA level of cell cycle inhibitor gene, RB and p53, resulting in up-regulation of Cyclin B and Cyclin E mRNA. However, this technique could not generate permanent gene knockdown.

Second, 3D cell culture technique was performed to establish shrimp cell culture. 3D cell culture is one of cell culture method that created an artificial environment for cell to grow and interact with their surrounding in all 3-dimensions. This method allow cell to grow within a closely mimic in vivo microenvironment which enhance natural cellular communication and signaling in cell. Shrimp cells were grown as 3D spheroid cell by using 96-well U-Shaped bottom microplate. 2 days of seeding, shrimp cells spontaneous self-assembled aggregation was detected. From Day 5 onward, multiple cells aggregated to form round spheroids with multiple cells around spheroids. Increase in spheroid size and cell proliferation were observed on day 7 and 9 of culturing, follow by significant decreased after 14 days. Unfortunately, the 3D spheroids could not maintain their viability for more than 21 days. Gene expression analysis of mitotic genes showed significant down-regulation of DLGAP5, CDK1, Cyclin H, and Plk1 after 2 days of culturing, but gene expression of these gene was recovered on day 7. Cyclin B was significantly up-regulated (2.0 – 3.5-fold change) in spheroid at 2- and 7- days of culturing. These results suggested that newly developed 3D shrimp cell culture provide interesting results to the recovery of mitotic related-genes corresponding to the increase in proliferation rate of spheroids at 7 days. Unfortunately, unlimited proliferation could not be achieved via 3D culture technique.

Lastly, chemical treatment with strong mutagen, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), was used to treated shrimp primary cell in an attempt to induce transformation leading to immortalized cell line. In this research work, the effect from the treatment of MNNG on shrimp primary cell were studied in 2D and 3D cell culture technique. In 2D cell culture, the ovary cell changed in their morphology from epithelial to round cells whereas epithelial cells could be observed in non-treated MNNG after 2 days of MNNG treatment. After 2 weeks, not only vast number of cell death were observed, but also fibroblast cell without nucleus were found in MNNG treated condition. Moreover, gene expression analysis of mitotic network genes (DLGAP5, Cyclin B, CDK1, CDK7, and Plk1) and telomere maintenance gene (NOP10) were significantly down-regulated in MNNG-treatment group when compare with ovary tissue. For 3D cell culture, ovary primary cells were cultured as 3D spheroid cell and treated with MNNG. The results showed that MNNG treatment in 3D spheroid cell could not increase the size and cell viability when compare to non-MNNG treated. The results of these studies suggested that unlimited proliferation of the 2D and 3D cultured cells did not occur via MNNG treatment.

In summary, the investigation results demonstrated that serveral genes may serve as potential targets for genetic manipulation aim to maintain cell proliferation and establishment of shrimp continuous cell line. Despite shrimp continuous cell culture establishment was not successful, gene expression of cell cycle relate gene from 3D cell culture demonstrate that the expression was restore toward cell proliferating. By utilizing these findings, we hope that it may lead to a successful immortalized cell line in the future.