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

[課程博士・論文博士共通]

博士学位論文要約
Summary

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論文題目 Title	Study on the establishment of continuous cell culture from Kuruma shrimp, *Marsupenaeus japonicus*		

This study attempts to establish the continuous cell line 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, which can help facilitate the control and prevention of shrimp disease. The development of shrimp cell culture has been studied over three decades since 1986 with many techniques such as medium and supplements used, transformation of primary cell with viral oncogenes, and cell fusion. However, no continuous shrimp cell lines have yet been successfully established. The lack of knowledge on the molecular level of shrimp cell under culture condition may be a major problem of development of shrimp cell culture. Therefore, the understanding the gene expression profile changes in shrimp primary cell and the investigation of the key regulator genes that control proliferation and cellular arrest of shrimp cell under culture condition is necessary.

This study attempts to establish the continuous cell line from kuruma shrimp, *Marsupenaeus japonicus*, which consists of two parts, investigation and establishment. First, investigate the molecular mechanisms that control shrimp cell proliferation and cellular arrest, shrimp primary cell culture was studied. The study starts with gene expression profiling of shrimp primary cells which aims to investigate the changes in gene expression profile between shrimp primary cells (*in vitro*) and shrimp tissue (*in vivo*), follows by differentially expressed genes (DEGs) analysis. This study aimed to comprehensively identify key regulator genes that control cell cycle progression in culture conditions. 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.

To comprehensively explore gene expression pattern of shrimp primary cell, 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*). These results provide further understanding on molecular mechanism and key molecular pathways of 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. However, there are many aspects of gene expression profile results which cannot be clearly explain may be due to many transcripts identified from RNA sequencing cannot be mapped to any known genes and their function is still unknown. Since many transcripts cannot be identified their function and pathway is also unknown, this may result in incomplete mapping of the pathways hence complete understanding of the gene expression profile is not possible. The gene functional and mechanism pathway would need to be further studies.

Next, the establishment of shrimp continuous cell line was attempted using various method including gene silencing via RNA interference (RNAi), chemical treatment, and 3-dimensional (3D) cell culture technology. Gene silencing method and chemical treatment were applied in an attempt to establish shrimp cell line by targeted cell cycle progression and checkpoints which may result in uncontrollable promotion of cell proliferation and led to immortalization of cell lines. In this study, gene silencing aim to inactivating p53 and

RB (cell cycle checkpoint genes) which may lead to establishment of shrimp immortal cell. Although the success of p53 and RB gene silencing and the enhancing of cell cycle progression reported in this study, Shrimp continuous cell line with unlimited proliferation could not be established. The limitation of RNAi technique was due to its mechanism which occurred on post-transcriptional level that target mRNA, RNAi via dsRNA will only has transient effect and could not generate permanent gene knockdown. Thus, shrimp primary cell should be continuously transfected with dsRNA to maintain the increase of cell cycle related genes.

Chemical treatment by using *N-methyl-N'-nitro-N-nitrosoguanidine* (MNNG) function by interfering the cell cycle control process with random point mutation on chromosome. Due to point mutation being generate at random location, the experiment cannot be easily control and may not be easily repeat. Moreover, strong alkylation of DNA from MNNG might cause the damage to be too severe for DNA to repair (more than 100-fold compare to other monofunctional alkylating agents) which lead to cell death. In the future study, lowering the concentration of MNNG treatment need to be further examine to lower cell mortality from MNNG toxicity but with high enough concentration to become carcinogenic leading to immortalization of the cell. Due to point mutations being generate at random after MNNG treatment, large scale screening may also be done to help identify cell with mutation interest faster.

Lastly, 3D cell culture method was used for the establishment of shrimp continuous cell culture. 3D cell culture system might stimulate growth of *in vitro* shrimp cell as same as *in vivo* condition by created an artificial environment for growing and enhance cell-cell communication. In this work, shrimp primary cells aggregated and formed round shape also known as spheroid cells. The shrimp spheroid cells showed high number of small cells surrounding the spheroid surface. Moreover, the 3D cell culture method could maintain spheroid cells viability for 21 days. However, the size and proliferation capability of shrimp spheroids were limited which eventually lead to the decreased in size which might cause by limited amount of oxygen and nutrition was unable to reach cells which located deep within the center of the spheroid. The proliferating cells could be found at the outer layer which are exposed to sufficient nutrients and oxygen on the outside, whereas cells at the core of spheroid was in quiescent and necrosis stage due to the lack of oxygen and nutrients. To overcome this problem, bioreactor has been reported to improve oxygen and nutrients transfer from medium pass through inner core cell. Furthermore, gene expression profile of shrimp spheroid cell was compared to shrimp tissue and 2D cell culture. Both mitotic maker genes expression result by qRT-PCR and whole gene expression pattern by RNA sequencing showed that gene expression profile of shrimp 3D spheroid cells was clustered into the same group with shrimp tissue (*in vivo*) and not with 2D cells. These results suggested that 3D cell culture system could accurately generates environment which closely mimic *in vivo* cell. This study utilized a new technique for generating shrimp cell culture as 3D spheroid cell. These results suggested that shrimp primary cell could form spheroid round cell with multi proliferating cell surround spheroid. The shrimp spheroid could survive for over 3-weeks. In addition, gene expression profile of shrimp spheroid cells was similar to shrimp tissue (*in vivo*). Unfortunately, shrimp continuous cell line could not be successfully generated via 3D cell culture technique but we suggested that shrimp spheroid cells can also be used as a model to study shrimp pathogens and a drug development.

In summary, the investigation results demonstrated that cell culture condition induced down-regulated of several genes that play role to promote or required for proliferating of shrimp cell. This finding suggested that restoration of expression of these genes may be potential 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.