MOLECULAR CLONING AND EXPRESSION STUDY OF *GROWTH-FACTOR RECEPTOR BOUND PROTEIN 7* (*GRB7*) GENE IN ZEBRAFISH DEVELOPMENT

TAN SHING CHENG

A project report submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2011

Acknowledgement

First of all, I would like to express my deepest appreciation and sincere gratitude to Dr. Lee Kui Soon for his advice, support and encouragement all this while. It has been an honour for me to work on this project under his supervision. His selfless guidance and comments have been a great help for me throughout this project.

I also wish to thank Assoc. Prof. Dr. Edmund Sim Ui Hang, Assoc. Prof. Dr. Hairul Azman Roslan, Dr. Ho Wei Seng and Dr. Samuel Lihan for providing me necessary equipments and reagents in this project. I wish to express my cordial appreciation to members of Proteomic Laboratory, Molecular Genetics Laboratory, Microbiology Laboratory, Virology Laboratory and Biochemistry Laboratory for allowing me to use the facilities in their laboratories.

I am grateful to post-graduate students in the Department of Molecular Biology, especially Xiang Ru, Wen Ni, Liyana, Jong Jen, Ngui Sing and Kathleen for their generous help throughout the course of my project. I wish to thank my coursemates in UNIMAS, especially Pey Ling, Chandra and Fatimah for their helpful suggestions. I am also indebted to the lab assistant, Mr. Iskandar, and to Kok Sin from UKM Medical Molecular Biology Institute for obtaining some essential materials in this project for me.

Special thanks to all my labmates in Animal Biotechnology Laboratory, especially Kim Loong, Louis, Ai Peng, Hua Sien, Sin Ling, and Winnie, for the stimulating discussions and for all the fun we have had in the laboratory.

Last but not least, I am thankful to all my family members for their love and support.

Declaration

I hereby declare that this dissertation is the result of my own work and no portion of this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

Signature : ……

Name : TAN SHING CHENG

Date : 25 MAY 2011

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List of Abbreviations

bp	Base pair
BPS	Between pleckstrin and Src homology
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EphB1	Ephrin type-B receptor 1
FAK	Focal adhesion kinase
G7-18NATE	Grb7-peptide18-No Arms Thioether
GM	Growth-factor receptor bound protein and Mig10
Grb7	Growth-factor receptor bound protein 7
HER2	Human epidermal growth factor receptor 2
hpf	Hour post-fertilization
IPS	Insert between the PH and SH2
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Insulin receptor
KCl	Potassium chloride
LB	Lysogeny broth
$MgCl_2$	Magnesium chloride

mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PH	Pleckstrin homology
PIR	Phosphotyrosine interacting region
Pro-rich	Proline-rich
PTP	Protein tyrosine phosphatase
RA	Ras-associating-like
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
SH2	Src homology 2
Shc	Src homology 2 domain containing transforming protein
SHPTP2	Src homology 2 domain containing protein-tyrosine phosphatase 2

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Molecular Cloning and Expression Study of *Growth-factor Receptor Bound Protein 7* (*Grb7*) Gene in Zebrafish Development

Tan Shing Cheng

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Grb7 is a multi-domain adaptor protein which has been postulated to play a role in the regulation of cell migration. The ability to regulate cell migration implies that the protein may be an important component in the progression of cancer as well as in the embryonic development of an organism. Therefore, this study aims to investigate expression pattern of *Grb7* throughout the development of zebrafish, whose cell migration process during embryonic development is well-understood. Semi-quantitative RT-PCR was employed to examine the pattern of *Grb7* expression in twelve stages of zebrafish development. The results showed that the gene was expressed in all of the stages studied, although the levels of expression differed among the stages. Comparisons with previous studies revealed that the temporal expression pattern of *Grb7* corresponded to that of some of its known binding partners, including PDGFR, EGFR and IR. This suggests the possible occurrence of interactions between Grb7 and these molecules during the process of development in zebrafish.

Keywords: gene expression, Grb7, molecular cloning, RT-PCR, zebrafish

ABSTRAK

Grb7 merupakan sejenis protein adaptor pelbagai domain yang dipercayai memainkan peranan penting dalam regulasi migrasi sel. Kebolehan untuk meregulasi migrasi sel menimbulkan kemungkinan bahawa Grb7 merupakan salah satu komponen yang penting dalam progresi kanser dan juga dalam perkembangan embryonik sesuatu organisma. Oleh itu, kajian ini dijalankan untuk menyiasat corak ekspresi Grb7 *dalam proses perkembangan ikan zebra, memandangkan proses migrasi sel yang berlaku pada perkembangan embryonik ikan tersebut telah jelas difahami. Kaedah RT-PCR semi-kuantitatif telah digunakan untuk memeriksa corak expresi* Grb7 *dalam 12 peringkat perkembangan ikan zebra. Hasil kajian ini menunjukkan bahawa ekspresi gen tersebut wujud pada kesemua peringkat perkembangan yang diperiksa, walaupun tahap ekspresi berlainan antara peringkat-peringkat tersebut. Perbandingan dengan kajian yang lepas menunjukkan bahawa corak ekspresi* Grb7 *bersepadanan dengan pasangan pengikatannya, termasuk PDGFR, EGFR dan IR. Ini mencadangkan kemungkinan berlakunya interaksi antara molekul-molekul ini dalam proses perkembangan ikan zebra.*

Kata kunci: ekspresi gen, Grb7, ikan zebra, kloning molekular, RT-PCR

1.0 Introduction

Growth-factor receptor bound protein 7 (Grb7) is an adaptor protein which plays an important role in linking various signalling molecules to their downstream effectors, leading to the activation of intracellular signalling pathways. In zebrafish, the protein is made up of 530 amino acid residues (Appendix A) and is encoded by the *Grb7* gene located on linkage group 24. The gene consists of 14 exons and spans a region of approximately 26.3 kilobase pairs.

As an adaptor protein, Grb7 lacks intrinsic enzymatic activity and is composed exclusively of motifs and domains. These include an N-terminal proline-rich (Pro-rich) motif, a ras-associating-like (RA) domain, a pleckstrin homology (PH) domain, a phosphotyrosine interacting region (PIR) domain, and a C-terminal Src homology 2 (SH2) domain (reviewed in Han *et al*., 2001). The presence of multiple motifs and domains indicates that the protein could have many binding partners and could therefore elicit complex biological functions, although the exact biological roles of Grb7 have not been determined.

However, it has been suggested that Grb7 may play a role in the regulation of cell migration, since the protein has been shown to interact with various tyrosine kinase receptors, proto-oncogenes and phospholipids, which play important roles in mediating the process of cell migration. Interaction of Grb7 with focal adhesion kinase (FAK), for example, has been shown to stimulate integrin-mediated cell migration (Han & Guan, 1999). In addition, alterations to its binding domains, which abolish the binding of the protein to certain tyrosine kinases and phospholipids, have been shown to eliminate its

ability in stimulating cell migration (Han *et al*., 2000; Shen *et al*., 2002). Moreover, the central part of Grb7 shares close structural homology with that of Mig10, a neuronal cell migration protein of *Caenorhabditis elegans* (Manser *et al*., 1997). This implies that there is a possible role of Grb7 in controlling cell migratory processes of vertebrates. The ability of Grb7 in regulating cell migration is further envisaged when the protein has been found to be co-amplified and overexpressed in a variety of invasive cancer cells (Kishi *et al*., 1997; McIntyre *et al*., 2005; Stein *et al*., 1994; Tanaka *et al*., 1998; Walch *et al*., 2004), which suggests that Grb7 may be able to promote the metastatic spread of cancer cells.

Since cell migration is an important event in embryonic development, expression study of *Grb7* in the development of an organism is likely to provide some hints to the biological functions of the protein. However, there is currently a lack of information on the expression pattern of the gene throughout an organism's development. Therefore, this study focuses on analysing the expression pattern of *Grb7* throughout the embryonic development of zebrafish, whose cell migratory process during development is well-characterised. Analysis of gene expression was performed at mRNA level on twelve stages of zebrafish development, namely the 4-cell (1.00 hpf), 1000-cell (3.00 hpf), dome (4.33 hpf), 50%-epiboly (5.25 hpf), 75%-epiboly (8.00 hpf), 1-somite (10.33 hpf), 14-somite (16.00 hpf), prim-5 (24.00 hpf), long-pec (48.00 hpf), protruding-mouth (72.00 hpf), first-feeding early larval (96.00 hpf), and adult (120.00 hpf) stages, by using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR).

The objectives of this study are:

- To clone the *Grb7* gene from zebrafish.
- To investigate the expression pattern of *Grb7* at mRNA level throughout the development of zebrafish.

2.0 Literature Review

2.1 Growth-factor receptor bound protein 7 (Grb7)

Growth-factor receptor bound protein 7 (Grb7) is a protein consisting of 532 amino acids in human, 535 amino acids in mouse, and 530 amino acids in zebrafish, according to the sequences deposited in Genbank (accession numbers: NM_005310, human; NM_010346, mouse; XM_003201342, zebrafish). In zebrafish, the protein is encoded by the *Grb7* gene located on linkage group 24, which consists of 14 exons and spans a region of approximately 26.3 kilobase pairs. Zebrafish Grb7 has a molecular weight of 53,374.48. The nucleotide sequence of zebrafish *Grb7* cDNA and the corresponding amino acid sequences are shown in Appendix A, and the structure of the protein is represented diagrammatically in Appendix B. This protein has been thought to be involved in the regulation of cell migration, since its central region shares close structural similarities with Mig10, which plays a role in long range migration of neuronal cells during *C. elegans* embryonic development (Manser *et al*., 1997). In addition, Grb7 has been shown to interact, primarily through its SH2 domain, with many growth factor receptors (see Section 2.3), which are important in mediating signal transduction in cell migration in response to certain growth factors or chemoattractants (Han *et al.*, 2001).

2.2 Structure of Growth-factor receptor bound protein 7 (Grb7)

Grb7 protein contains a proline-rich (Pro-rich) motif, in addition to four binding domains, namely a Ras-associating-like (RA) domain, a pleckstrin homology (PH) domain, a phosphotyrosine interacting region (PIR) domain, and a Src-homology 2 (SH2) domain (Han *et al*., 2001; Figure 1). The Pro-rich motif is located at the amino (NH_2) terminal region of the protein, while the SH2 domain is located at the carboxyl (COOH) terminal

region (Han *et al.*, 2001). The RA and PH domains, on the other hand, are located at the central region of Grb7 protein (Han *et al.*, 2001). These two domains are embedded in a region of about 300 amino acids, which shares close structural homology with the central part of a *Caenorhabditis elegans* cell migration protein, Mig10, and this region is therefore known as the GM (Grb-Mig10) region (Manser *et al.*, 1997). The PIR domain is located between the PH and SH2 domains (Kasus-Jacobi *et al.*, 2000).

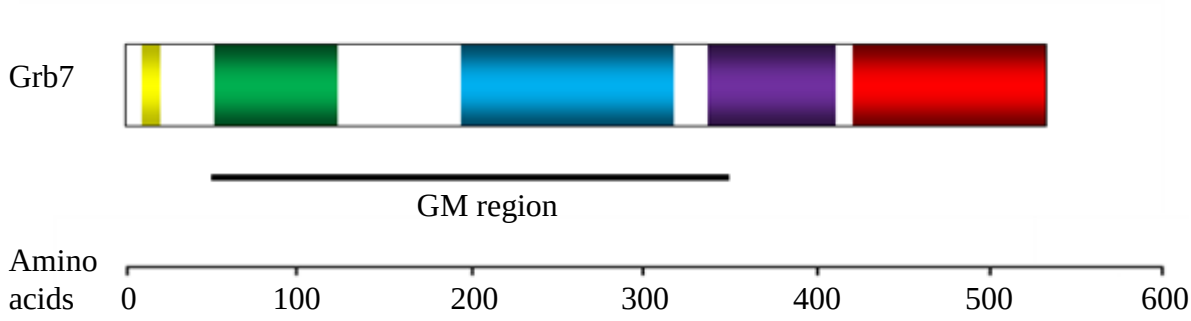

Figure 1: Domain structure of Grb7. Each motif or domain is represented by a different colour: yellow, Pro-rich motif; greesn, RA domain; blue, PH domain; purple, PIR domain; red, SH2 domain. The RA and PH domains are embedded in the GM region. The scale represents the size in amino acids.

2.2.1 Proline-rich (Pro-rich) motif

The Pro-rich motif of Grb7 consists of a highly conserved sequence of 10 amino acid residues located at the NH_2-terminus of the protein. Based on the sequences deposited in Genbank, the amino acid sequence of this domain in zebrafish Grb7 protein is PLIPNPFPEL. This shares a 90% sequence homology with that of human and mouse Grb7, which is PSIPNPFPEL. The Pro-rich motif runs from amino acids 69 to 78 in zebrafish, 65 to 74 in human, and 64 to 73 in mouse and rat. The first four amino acid residues in this motif conforms to the PXXP consensus sequence for interaction with src-homology 3 (SH3) domain-containing proteins (Yu *et al.*, 1994). Thus, the presence of Pro-rich motif in Grb7 indicates the possibility that this protein may interact with such and other signalling molecules. However, no protein has yet been demonstrated to interact with the Pro-rich motif of Grb7 (Han *et al.*, 2001).

2.2.2 Ras-associating-like (RA) domain

The RA domain contains approximately 80 amino acid residues. The amino acid sequences of the RA domain in human and mouse Grb7 are highly conserved, with at least 90% sequence homology, and these share about 50% homology with that of zebrafish Grb7. Although it has been suggested that Grb7 may be involved in the regulation of Ras signalling pathways through its RA domain, the functional role of this domain in Grb7 is still incompletely understood (Han *et al*., 2001). This is because a number of experiments conducted initially had detected no interaction between Grb7 and G-protein (Leavey *et al*., 1998), and it was until recently that the binding of full-length Grb7 to Ras has been reported (Rodriguez-Viciana *et al*., 2004).

2.2.3 Pleckstrin homology (PH) domain

PH is a domain of approximately 100 amino acid residues, which was first detected in 1993 in the protein pleckstrin (Haslam *et al*., 1993). This domain has subsequently been shown to present in many other proteins involved in intracellular signalling (Gibson *et al*., 1993; Mayer *et al*., 1993). The ligand binding site of PH domains is made up of a positively-charged variable region (Lemmon *et al*., 1996). The domain therefore targets negatively-charged molecules such as phospholipids, and less commonly, other proteins involved in signal transduction (Flynn, 2001). It has been suggested that these interactions induce conformational changes of the host protein, which facilitate the process of phosphorylation, leading to downstream signalling pathways (Toker & Newton, 2000).

In Grb7, the PH domain has been shown to mediate the interaction between the protein and phosphatidylinositol phosphates. Intentionally-induced mutations to the Arg^{239} of the critical arginine/lysine residue abolish the binding of Grb7 to phosphatidylinositol

phosphates, suggesting that the mechanisms by which Grb7 PH domain mediate interactions with phosphatidylinositol phosphates is similar to those of other PH domains (Shen *et al*., 2002). Binding of Grb7 to phosphatidylinositol phosphates through its PH domain allows the protein to be phosphorylated by the focal adhesion kinase (FAK), which is necessary for the regulation cell migration (Shen *et al*., 2002).

2.2.4 Phosphotyrosine interacting region (PIR) domain

The PIR domain is comprised of about 50 amino acid residues. This domain is located between the PH and SH2 domains and hence, is also referred to as the Between PH and SH2 (BPS) domain or the Insert between PH and SH2 (IPS) domain. The PIR domain has been shown to affect the binding specificity of SH2 domain-mediated interactions with receptor tyrosine kinases (He *et al*., 1998). However, the mechanism by which the PIR domain influences the above-mentioned binding specificity is not well understood. In addition, the domain has also been shown to mediate the binding of its host proteins to the kinase domains of activated insulin receptor (Kasus-Jacobi *et al*., 1998) and insulin-like growth factor 1 (IGF-1) receptor (He *et al*., 1998). In Grb7, the PIR domain, together with the SH2 domain, is implicated with the binding to insulin receptor, although the SH2 domain appears to play a more important role in this interaction (Kasus-Jacobi *et al*., 2000).

2.2.5 Src-homology 2 (SH2) domain

The SH2 domain consists of approximately 100 amino acid residues which fold into a structural configuration of βαββββββαβ (Pawson *et al*., 2005). This domain is non-catalytic and has been found in a variety of cytoplasmic signalling proteins (Janes *et al*., 1997). In addition to its role as an adaptor between receptors and downstream signalling molecules, SH2 domain also functions in intracellular signal transduction and in the regulation of

kinase activity of specific proteins (Pero *et al*., 2002). Besides, along with SH3 and pleckstrin homology domains, SH2 domain also mediates inter- and intramolecular interactions involved in signal transduction from activated receptor tyrosine kinases (Cohen *et al*., 1995; Pawson, 1995).

In Grb7, SH2 domain has been shown to mediate interactions with specific phosphotyrosines contained within pYXN sequence motif in autophosphorylated receptor tyrosine kinases as well as other tyrosine phosphorylated signalling molecules (Daly, 1998; Han *et al*., 2001). The specificity of these interactions is affected not only by the amino acid sequences of the SH2 domains, but also those flanking the phosphotyrosine (Daly, 1998). The functional importance of this domain in Grb7 was highlighted when a splice variant of human Grb7, which lacks SH2 domain, was shown to constitutively activate signal transduction pathways, since the protein was irresponsive to epidermal growth factor (EGF) stimulation and was not dephosphorylated even in serum-starved quiescent cells, due to the lack of interaction with EGF receptor and protein tyrosine phosphatase (PTP), respectively (Tanaka *et al*., 1998). As a result, the expression of the Grb7 splice variant contributed to the invasive and metastatic progression of human oesophageal carcinomas (Tanaka *et al*., 1998).

2.3 Binding partners of Grb7

Grb7 has been shown to interact with various proteins, primarily through its SH2 domain. The SH2 domain-mediated protein-protein interactions include epidermal growth factor receptor (EGFR) (Margolis *et al*., 1992), human EGF receptor 2 (HER2) (Stein *et al*., 1994), Src homology 2 domain containing (Shc) transforming protein (Stein *et al*., 1994), Src homology 2 domain containing protein-tyrosine phosphatase 2 (SHPTP2) (Keegan &

Cooper, 1996), platelet-derived growth factor receptor (PDGFR) (Yokote *et al*., 1996), Ret proto-oncogene (Pandey *et al*., 1996), ErbB3 (Fiddes *et al*., 1998), c-Kit (Thömmes *et al*., 1999), focal adhesion kinase (FAK) (Han & Guan, 1999), Tek/Tie2 (Jones *et al*., 1999), caveolin (Lee *et al*., 2000), insulin receptor (IR) (Kasus-Jacobi *et al*., 2000), Rnd1 (Vayssiere *et al*., 2000), and ephrin type-B receptor 1 (EphB1) (Han *et al.*, 2002). It should be noted that although the SH2 domain is the major mediator in the interaction between Grb7 and IR, this interaction can also occur through the PIR domain (Kasus-Jacobi *et al*., 2000). In addition, the RA domain is implicated with binding of Grb7 to Ras (Rodriguez-Viciana *et al*., 2004), and the PH domain is involved in the interaction with various phospholipids, particularly phosphatidylinositol-3-phosphate and phosphatidylinositol-5-phosphate (Shen *et al*., 2002).

2.4 Normal expression and subcellular localisation of *Grb7*

In murine, *Grb7* is highly expressed in kidney (Margolis *et al*., 1992), liver (Margolis *et al*., 1992) and gut (Leavey *et al*., 1998). The specific localisation of *Grb7* expression at mRNA level in kidney include the proximal convoluted tubule, proximal straight tubule, thin descending limb, medullary thick ascending limb, cortical thick ascending limb, cortical collecting duct, outer medullary collecting duct, and inner medullary collecting duct (Leavey *et al*., 1998). No expression was found in the arcuate artery and glomerulus (Leavey *et al*., 1998). Apart from kidney and liver, expression of *Grb7* was also found in murine testis and ovary, although in a lower level (Margolis *et al*., 1992). By exposing various tissues to ^{32}P-labeled probe for a long period of time in an RNA blot analysis, *Grb7* was also found to be weakly expressed in murine lung, but not in the spleen, brain, heart and muscle (Margolis *et al*., 1992).

The *Grb7* expression pattern in human differs slightly from that of murine, in that the expression in human is found in tissues from a broader range of organs. The gene is strongly expressed in the pancreas, kidney, placenta, prostate and small intestine, and is weakly expressed in the colon, liver, lung and testis (Frantz *et al*., 1997). Besides, very low level of expression was detected in the human thymus and peripheral-blood leukocytes (Daly, 1998).

The specific localisation of gene expression within a cell has been determined by immunofluorescence staining on Chinese hamster ovary (CHO) cells. This revealed that the localisation of Grb7 protein is at the cytoplasm and discrete regions of the plasma membrane called focal contacts (Han *et al*., 2000).

2.5 Roles of Grb7 in cell migration

The first indication that Grb7 may be involved in the regulation of cell migration came from the fact that the protein shares a close structural similarity with Mig10, which plays a role in long range migration of neuronal cells during embryonic development. In addition, Grb7 has been shown to interact with many growth factor receptors (see Section 2.3), which are important in mediating signal transduction in cell migration in response to certain growth factors or chemoattractants. The role of Grb7 in cell migration was proven when transient transfection of CHO cells with expression vectors encoding various fragments of Grb7 stimulated their migration (Han *et al*., 2000). Besides, deletion of the Grb7 SH2 domain (Han *et al*., 2000) as well as mutation to its PH domain (Shen *et al*., 2002) have both been shown to eliminate the protein's ability to stimulate cell migration.

2.6 Roles of Grb7 in cancer

The ability of Grb7 to stimulate cell migration can be made use by cancer cells to promote their invasion and metastatic spread. This is particularly true when the chromosomal localisation of *Grb7* has been mapped to a mouse chromosome 11 locus syntenic to a region of human chromosome 17q, which contains the gene encoding the HER2 that is commonly amplified in breast cancer (Daly, 1998). Therefore, *Grb7* has been found to be co-amplified and overexpressed with *HER2* in a number of breast cancer cell lines as well as primary breast cancer samples (Stein *et al*., 1994). In addition to breast cancer, *Grb7* has found to be co-expressed with *HER2* in human oesophageal carcinoma (Tanaka *et al*., 1998), gastric carcinoma (Kishi *et al*., 1997), high grade intraepithelial neoplasia (Walch *et al*., 2004) and invasive Barrett's carcinoma (Walch *et al*., 2004). The gene has also been found to be overexpressed in primary testicular germ cell tumor samples and cell lines (McIntyre *et al*., 2005).

The role of Grb7 in the progression of cancer is further envisaged when it has been shown that expression of antisense Grb7 RNA can suppress the invasive phenotype of oesophageal carcinoma cells *in vitro* (Tanaka *et al*., 1998). In addition, an inhibitory peptide, Grb7-peptide18-No Arms Thioether (G7-18NATE), which binds specifically to Grb7 SH2 domain, has been shown to be able to prevent cancer cell proliferation and migration (Pero *et al*., 2002). Taken together, these findings indicate that Grb7 can contribute to the metastatic spread of cancer cells.

2.7 Zebrafish (*Danio rerio*)

The zebrafish, *Danio rerio* (formerly also known as *Brachydanio rerio*), is a teleost belonging to the family *Cyprinidae*. The fish is native to the rivers of the Indian, Pakistan, Nepal and Bhutan subcontinents (Dahm *et al*., 2005; van der Sar *et al*., 2004), where the water is relatively stagnant or slow-moving with a temperature of 27℃ to 34℃ and a pH of 7.9 to 8.2 (Engeszer *et al*., 2007).

Zebrafish possess three classes of pigment cells, namely melanophores, xanthophores and iridiophores, which contribute to black, yellow and silver colours, respectively. Morphologically, male zebrafish appear longer and narrower than the females, and are also more reddish in colour (Dahm *et al*., 2005). In addition, female zebrafish possess a larger belly for accommodation of their eggs. Zebrafish can grow up to 2.5 inches in size, although they are often less than 4 centimeters long in captivity. Their life-span is around 2 to 3 years, although this may extend to 5 years under ideal growth conditions (Spence *et al*., 2008).

The zebrafish genome is 1.7 gigabases in size, and is divided into 25 linkage groups. Current prediction from the eighth integrated whole genome shotgun assembly of zebrafish genome (Zv8) released on Ensembl (http://www.ensembl.org/Danio_rerio/Info/Index) suggests that the genome is comprised of 24,147 protein-coding genes, 80 pseudogenes, and 6 retrotransposed genes.

2.7.1 Zebrafish as a model in developmental biology

Zebrafish has emerged as a model organism for various biological research, including the modeling of human disease (Barut & Zon, 2000), functional genomics and therapeutic

development (Nasevicius & Ekker, 2001), oncology (Amatruda *et al*., 2002), pathology (Neely *et al*., 2002), immunology (Traver *et al*., 2003), toxicology (Hill *et al*., 2005), regenerative medicine (Major & Poss, 2007; Tawk, *et al*., 2002), and most notably, developmental biology (Dahm *et al*., 2005). The fish serve as a real-time *in vivo* model for studying the expression pattern of developmentally regulated gene, and developmental biology experimentations by using the fish have provided many important insights into the formation and function of individual tissues, organs and neural networks (Liang *et al*., 2009).

There are many advantages of using zebrafish as a model of developmental biology research. First, the embryos develop externally and are transparent, making the visualisation of its internal organs possible. Second, mating can be induced easily by adjusting light conditions, allowing each female to lay up to 200 eggs per week (Dahm *et al*., 2005). In addition, a diverse range of molecular tools and methods are available for developmental studies on zebrafish. The embryonic development of the fish is also rapid, facilitating researchers to follow the entire course of development. Apart from that, the fish shares many orthologous genes with mammals, and large regions of their chromosomes show synteny to that of humans (Barbazuk *et al*., 2000), which imply that biological processes occurring in the fish may be similar to those of the other higher organisms.

However, one disadvantage of using zebrafish as a model in developmental biology is that paralogous genes can be present in the fish genome, since part of euteleost genome has been duplicated during evolution more than 100 million years ago (Amores *et al*., 1998). This often makes the comparison of homologous developmental pathways difficult.

2.7.2 Developmental stages of zebrafish

The developmental stages of zebrafish have been described by Kimmel *et al*. (1995) based on the morphological features of the embryos. This divides zebrafish development into 34 different stages, and these stages can be grouped into seven developmental periods, namely zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. The developmental stages can be estimated by the point of time after fertilisation has occurred (hour post fertilisation, hpf), as shown in Appendix C.

3.0 Materials and Methods

3.1 Fish stocks

Zebrafish were obtained from the fish facility in the Department of Molecular Biology, Universiti Malaysia Sarawak. The fish were maintained at approximately 28.5 ℃, under a 14-hour light/10-hour dark photoperiod as described by Westerfield (1995). All embryos were collected by natural spawning, and kept in the E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$). The embryos were then dechorionated manually and staged according to Kimmel *et al*. (1995).

3.2 Isolation of total RNA

Total RNA was isolated from twelve stages of zebrafish development, namely the 4-cell (1.00 hpf), 1000-cell (3.00 hpf), dome (4.33 hpf), 50%-epiboly (5.25 hpf), 75%-epiboly (8.00 hpf), 1-somite (10.33 hpf), 14-somite (16.00 hpf), prim-5 (24.00 hpf), long-pec (48.00 hpf), protruding-mouth (72.00 hpf), first-feeding early larval (96.00 hpf), and adult (120.00 hpf) stages, by using TRIzol® Reagent (Invitrogen, USA). First, approximately 100 embryos of each developmental stage were homogenised in 1 ml TRIzol® Reagent and centrifuged at 12,000 rpm for 10 minutes at 4 ℃. The supernatant obtained was transferred into a new tube and incubated at room temperature for 5 minutes. A total of 200 µl chloroform was then added into it. Following incubation at room temperature for 3 minutes, the tube was centrifuged at 12,000 rpm for 15 minutes at 4 ℃. The aqueous phase formed was transferred into a fresh tube containing 500 µl absolute ethanol and incubated at room temperature for 10 minutes, followed by centrifugation at 12,000 rpm for 10 minutes at 4 ℃. The supernatant formed was discarded and 1 ml 70% ethanol was added to the tube. Centrifugation was then performed at 7,500 rpm for 5 minutes at 4 ℃. The RNA pellet